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How does developmental plasticity differ between diverse types of nutrition regimes?

Mestrado em Biologia Evolutiva e do Desenvolvimento

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Abstract

Both developmental nutrition and adult nutrition affect life history traits, however little is known about the importance of specific nutrients relative to calories and the effects on adult traits such as lifespan or fecundity. The architecture of dietary requirements can respond to selection with a microevolutionary potential, but it is not clear whether these effects are due to caloric composition or due to the concentration and combination of macronutrients. Using *Drosophila melanogaster,* I compared the effects of different types of larval diets, differing in calories, protein, and carbohydrate content, and how they modify the response of both larval and adult life history traits. Using our standard lab medium as control, with a protein: carbohydrate (P:C) ratio of 1:10 and a caloric content of 1.44 kcal/ml, I diluted the protein content of the larval diet in two ways: either caloric dilutions maintaining a constant P:C ratio (1:10) but reducing caloric content to 0.72, 0.36, 0.18 and 0.09 kcal/ml, or macronutrient restriction where I substituted the calories from protein with carbohydrates from sucrose to generate four isocaloric diets (1.44 kcal/ml) that varied in their P:C ratios, 1:25, 1:50, 1:100 and 1:200. I reared larvae from an outbred population on each of these diets and assessed the effects on larval and adult traits. Larval traits included survival and developmental time from egg to pupae, development time in the second and third instar, and larval mouth hook length. For adults, I measured the size of adult organs, lifespan, and early fecundity. Overall, I found that macronutrient restriction generated more severe phenotypes, increasing development time and decreasing survival and mouth hook size more than caloric restriction. The two diet types produced animals of different shapes, with body parts differing in their least squared means and the slopes of their reaction norms in a complex manner with diet type. The larval diet types differed dramatically in their effects on lifespan; macronutrient restriction significantly shortened lifespan while caloric restriction dramatically lengthened lifespan. Finally, macronutrient restriction of the larval diet reduced early fecundity more than caloric restriction at the same protein concentration. Our results demonstrate that juvenile nutrition impacts both larval and adult life history traits, and is capable of modulating adulthood. Further, our data shows that altering the composition of the juvenile diets by caloric versus macronutrient restriction induces different responses in the life history traits analyzed, demonstrating both the importance of diet in juvenile development and its role in adult performance.

Keywords: developmental nutrition, caloric restriction, macronutrient restriction, phenotypic plasticity, life history traits

Resumo

A composição do substrato é muitas vezes o fator mais limitante quando se fala em *stress* nutricional porque os indivíduos precisam de regular muito bem a ingestão dos nutrientes presentes na comida para a manutenção da sua homeostasia. Os macronutrientes são essenciais para formação e manutenção de tecidos e para processos metabólicos e os animais têm de compensar a sua falta através de alocação de recursos ou decisões baseadas no gasto desses recursos em atividades indispensáveis para a sobrevivência.

Os animais regulam muito bem a ingestão de determinados alimentos uma vez que a qualidade nutricional é essencial ao seu desenvolvimento. Assim, fazem escolhas específicas refletindo as necessidades, a fase do ciclo de vida ou o seu ambiente. Diferentes estratégias de comportamento foram desenvolvidas consoante as necessidades de cada espécie e a nutrição e escolha perante determinadas opções alimentares podem desempenhar um papel importante no desenvolvimento e evolução das populações, com potencial microevolutivo. Desde que os animais dispersaram e conseguiram migrar para quase todos os habitats possíveis que as espécies estão adaptadas para mudar o seu índice nutritivo, demonstrando plasticidade fenotípica para se adaptarem.

Diferentes espécies têm diferentes necessidades nutricionais o que pode levar a alterações de comportamento na ingestão e alocação dos mesmos. Assim os animais, dependendo das suas necessidades, acabam por alterar a perceção do seu ambiente e consequentemente as estratégias a utilizar, tanto ao nível fisiológico como metabólico, para alcançar exigências nutritivas quando os nutrientes são limitados. Esta procura específica por determinado(s) nutriente(s) pode depender de fatores genéticos, ambientais ou da fase específica do ciclo de vida dos indivíduos, já que estes alteram a qualidade e a quantidade de nutrientes, dependendo da altura do desenvolvimento em que se encontram.

O consumo controlado de comida tem no entanto alguns benefícios, variando num grande leque de animais taxonomicamente diversos, desde leveduras aos primatas. Tradicionalmente, os efeitos positivos de alguma restrição alimentar foram atribuídos a facto do animal ingerir menos calorias, fazendo uma restrição calórica, uma convenção que ainda levanta alguns problemas. Devido ao carácter nutricional instável de cada substrato, devido entre outras coisas à ação de microrganismos, o balanço dos nutrientes está também em constante mudança. As preferências nutritivas que revelam são geralmente expressas pelo consumo dos dois macronutrientes principais, proteínas e

hidratos de carbono, tentando sempre encontrar um equilíbrio, ou rácio, entre ambos que melhore o mais possível a performance. Quando confrontados com situações de poucos recursos e nas quais têm de atingir o seu alvo nutricional otimizado os animais ponderam quais as escolhas possíveis nesse espaço de modo a atingir a melhore quantidade de substrato para o seu desenvolvimento, não só do ponto de vista de ingestão calórica mas também do consumo específico de macronutrientes importantes para o desenvolvimento e sobrevivência. Deste modo têm dois cenários possíveis: (1) quando um dos nutrientes se revela mais importante, a quantidade de comida ingerida é regulada de modo a atingir os níveis ótimos desse nutriente, ingerindo o outro em excesso ou (2) a ingestão em níveis intermédios de ambos os nutrientes. Por estas razões é importante haver estudos que separem bem o efeito entre quais as consequências de ingestão reduzida de calorias ou, mais especificamente, da ingestão de dietas em que foram reduzidos apenas um dos macronutrientes essenciais, deixando o conteúdo calórico intacto.

O alvo nutricional varia também ao longo da vida do animal sendo muito específico consoante a sua fase do ciclo de vida. Por exemplo quando a mosca da fruta, *Ceratitis capitata*, está perto da metamorfose o seu alvo nutricional deixa de ser maioritariamente proteico e passa a conter alto teor de hidratos de carbono que tem a energia necessária para a fase imediatamente antes da metamorfose. Após o acasalamento as fêmeas de *Drosophila melanogaster* aumentam a sua produção de ovos precisando de uma dieta mais rica em proteínas do que as fêmeas virgens.

Muitos exemplos na literatura deixam claro que há uma enorme importância e relação entre as calorias ingeridas, a qualidade nutricional medida pelo rácio dos dois macronutrientes principais e a fase de desenvolvimento em que os animais se encontram. Com este trabalho tentámos compreender quais as consequências para o adulto, quando são impostas restrições nutritivas apenas no desenvolvimento juvenil. Com esta finalidade usámos o modelo animal por excelência em estudos nutricionais e de adaptação fenotípica e comportamental, a mosca do vinagre, *Drosophila melanogaster.* Com este projeto pretende-se observar quais as modificações dos indivíduos adultos quando alterada apenas a alimentação juvenil e como conseguem alocar os recursos necessários quando têm restrições num dos macronutrientes fundamentais, tentando construir assim uma "base de dados fenotípica" de larvas e adultos em diferentes dietas. Com a importância dos ambientes nutricionais no metabolismo juvenil, que pode traduzir-se em síndromes metabólicas, com consequentes implicações no desenvolvimento, é de grande interesse

entender as correlações que influenciam as alterações morfológicas ou comportamentais, condicionando o normal crescimento do organismo com pressões nutritivas diferentes.

As características avaliadas, ou *life history traits*, são conhecidas por serem influenciadas por diferentes ambientes nutricionais. Nas larvas analisámos o tempo de desenvolvimento e a sobrevivência de ovo a pupa uma vez que ambos são afetado pela nutrição. Ainda na fase larval analisámos o cumprimento dos ganchos que fazem parte da estrutura da boca, e variam de tamanho entre os três estágios larvares. Estas estruturas devido à sua plasticidade foram escolhidas porque respondem às diferenças nutricionais alterando-se para permitirem a ingestão de substratos mais duros ou mais diluídos. Como a *Drosophila melanogaster* é um inseto holometabólico, sofre uma metamorfose complexa antes da fase adulta onde todos os tecidos e órgãos são restruturados e dão origem aos tecidos e órgãos do adulto. Depois da metamorfose os indivíduos param o seu crescimento, ou seja o tamanho do adulto é definido na fase da larva. Assim o tamanho do corpo do adulto foi analisado, através da dissecação de quatro estruturas, asas, fémur, tórax e o palpo maxilar, para perceber qual a influência da alimentação juvenil nesta restruturação dos tecidos. Nos adultos, nestes casos apenas em fêmeas, foram ainda analisadas a longevidade e a fecundidade, já que estas duas características importantes são normalmente inversamente proporcionais, visto que menor fecundidade leva a maior longevidade e vice-versa, bem como altamente dependentes da qualidade nutricional.

Para conseguirmos desvendar a influência dos macronutrientes e das calorias ingerida fornecemos aos indivíduos oito dietas diferentes, conseguidas a partir da comida controlo usada naturalmente em laboratório, com um rácio de proteína por hidratos de carbono (P:C) de 1:10 e com 1.44 quilocalorias por mililitro (kcal/ml). Das diferentes dietas, quatro representaram restrições calóricas, 0.72, 0.36, 0.18 e 0.09 kcal/ml, e as restantes quatro foram dietas isocalóricas, na qual apenas se alterou o rácio de P:C, 1:25, 1:50, 1:100 e 1:200.

Os nossos resultados demonstram, em relação às características analisadas para as larvas a desenvolverem-se nas oitos diferentes dietas, que o tempo de desenvolvimento de ovo a pupa foi muito aumentado, quando comparadas as dietas entre tratamentos de restrição calórica e restrição de proteína. Em todas as dietas com diferentes rácios de P:C o desenvolvimento foi tendencialmente mais demorado, com o exemplo mais significativo

na dieta de 1:200, com um tempo médio de aproximadamente 400 horas. Nas restrições calóricas o aumento mais extremo observou-se na diluição de 0.09kcal/ml e foi de aproximadamente 200 horas.

Nas análises de sobrevivência de ovo até pupa, os resultados foram mais significativos no rácio de 1:200 e verificámos que 20% dos indivíduos sobrevive, apresentado uma taxa de sobrevivência maior do que inicialmente previsto. O resultado mais parecido com este, nas restantes dietas analisadas, foi na diluição de 0.09kcal/ml cuja taxa de sobrevivência foi de 45%, subindo até chegar à diluição controlo. Ainda nas características larvares, o comprimento dos ganchos das bocas das larvas medido apenas nas larvas nos estágios L2 e L3, não foi muito diferente do controlo com diferenças muito pequena de tamanho entre todas as dietas.

No caso dos adultos, para as características morfológicas, encontrámos diferenças significativas nos quatro órgãos analisados. Todas as dietas com menos proteínas e calorias apresentam um menor tamanho nas dietas mais extremas, de ambos os tratamentos, com valores muito parecidos entre si e significativamente mais pequenos que as dietas controlo. Nos resultados de longevidade e fecundidade, as observações foram de encontro ao esperado. No rácio 1:200 as fêmeas puserem poucos ovos nos 7 primeiros dias e viveram significativamente menos que as fêmeas controlo, começando a morrer no dia 9 com a última mosca viva registada no dia 14. Já no rácio de 1:50 e nas duas dietas diluídas, 0.36 e 0.09 kcal/ml, as fêmeas embora tenham posto menos ovos que na comida controlo, viveram mais tempo do que as moscas no rácio *standard* de 1:10.

Estes resultados demonstram bem que a alimentação juvenil tem efetivamente um impacto no desenvolvimento e performance reprodutiva do adulto. Também é possível concluir que os macronutrientes, mais especificamente as proteínas, influenciam toda a vida do animal, mesmo sendo um fator limitante no desenvolvimento juvenil. No entanto uma das grandes conclusões a retirar deste projeto é também o facto de, para a *D. melanogaster,* o desenvolvimento juvenil em dietas com mesma quantidade de proteína mas com diferentes quantidades de calorias, faz variar as tendências das características fenotípicas e morfológicas analisadas o que é bem demonstrativo das diferenças entre fazer uma restrição calórica ou uma restrição de um macronutriente específico, no caso, a proteína. Isto significa que não é apenas este nutriente que é importante na dieta mas o rácio de proteína:carbohidratos, ou a proteína no contexto da quantidade de hidratos de carbono. Isto é especialmente verdade nas análises de longevidade dos adultos em que os fenótipos dos dois tratamentos vão em direções opostas.

Palavras – chave: desenvolvimento juvenil, nutrição, restrição calórica, restrição de macronutrientes, plasticidade fenotípica.

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1. Introduction

An animal's fitness depends on the quality of the resources at its disposal throughout its lifetime. The acquisition of nutrients is fundamental for the maintenance of bodily functions, growth, survival, lifespan, metabolism, and fecundity in all animals studied to date [1]. Altering the nutritional environment of developing animals generates variation in the morphology, life history and behavior. This phenomenon is known as developmental plasticity and these alterations often have lifelong effects. High profile studies in humans suggest that caloric deprivation in pregnant mothers causes changes in the developing fetus that have long-term effects on the metabolism and health of adults [2][3]. More recent studies from a wide range of animals suggest that in addition to calories, a more complex interplay between macronutrients in the diet regulate life history traits [4][5][6].

In this study, I used the fruit fly *Drosophila melanogaster* as a model to understand how different types of nutritional stress in larvae affect life history traits in the larval and adult stages. Several authors have shown that life history traits respond not only to the caloric composition of the food, but also to its balance of macronutrients [5][7][8]. Indeed, lifespan in adult insects appears more affected by the ratio of protein and carbohydrate in the adult diet than to its caloric content [9]. Nutrition in larval stages affects a number of life history traits, including development time, survival to pupa, starvation resistance, adult body size, and reproductive potential [10] Animals facing periods of nutritional stress must continue to grow and develop despite the quality or quantity of the food. How the effects of caloric restriction and macronutrient restriction in the juvenile diet translate into alterations in life history traits in adults remains unexplored. Hypothesizing that the plastic response to larval nutritional stress would be associated with trade-offs in adult fitness, I compared the consequences of modifying larval diets either via caloric or macronutrient restriction on larval and adult life history traits. By only changing the nutritional intake in larval life and allowing the adults to develop in a normal nutritional environment, I intend to address the life-history consequences of the plastic response to larval nutritional stress.

1.1 – Caloric Restriction versus Macronutrient Restriction– Definitions, Effects, and Caveats

In its most simple form, nutrition can be seen as the intake of energy, or calories. Commonly referred to as "undernutrition without malnutrition", limiting the total food

intake appears to be the consensual definition of caloric restriction, but this one has its own caveats since it is still unclear what “undernutrition” and “normal” food conditions are [11]. A long-standing problem in nutritional biology is to understand how caloric restriction is sensed and integrated by the animal, and how it affects the mechanisms regulating longevity.

The implementation of caloric restriction relies heavily on patterns of feeding behavior. In *Caenorhabditis elegans,* the most common method of dietary manipulation takes advantage of animals that are defective in pharyngeal constrictions — the *eat* mutants [12]. The food source, the bacterium Escherichia coli, is provided in abundance, but ingestion is limited by the neuromuscular defect of the mutants. In experiments with rodents, calorically restricted animals are fed a fraction (65% to 70%) of the food consumed by the *ad libitum* group [13]. Therefore, in both of these model systems caloric restriction relies on an overall reduction of nutrient intake, defined by the rates of consumption of the animal. These studies conclude that this reduction leads to partially or completely suppressed reproduction, increased resistance to oxidative stress, and increased longevity [14]. In contrast, in other systems caloric restriction typically involves a simple dilution of the food medium. This raises a long-standing problem in nutritional biology regarding both how to define caloric restriction but also to understand whether these different methods of caloric restriction would be regulated in the same manner.

Studies in *Drosophila melanogaster,* a key model organism for dissecting the effects of nutrition on life history, demonstrate that nutrition regulates many complex life history traits such as lifespan or ageing [11][13]. Flies are typically reared on nutrient rich diets, composed of a mixture of sucrose, cornmeal, molasses, and yeast extract that itself contains a variety of nutrients, lipids, vitamins and other small molecules. The majority of the caloric restriction protocols in *D. melanogster* employ the dilution of all dietary components at once, without altering the volume of food offered. Importantly, this method does not control for the fact that the animal might alter its ingestion rate in response to the reduced caloric content of the diet. Here, I will term this type of dietary restriction caloric restriction and for practical reasons will use this method in my studies.

More recent studies have questioned the role of calories in regulating life history traits, and propose that the quantities and balance of macronutrients also play important roles [15]. Animals regulate their macronutrient intake often by feeding on multiple food sources and try, when possible, to compensate for deficiencies in nutrients [16]. Not only do animals regulate the quantities of nutrients that they ingest, they often strive for

specific combinations, or ratios, of macronutrients like protein and carbohydrates [6][17]. Macronutrient restriction, in particular diluting the protein content of the food with carbohydrates, has been shown to affect a range of life history traits from stress resistance to cancer prevention of a taxonomically disparate organisms ranging from yeast to humans [18][19][20]. Unlike caloric restriction, macronutrient restriction alters the quantity of specific nutrients in the diet while maintaining the same caloric content.

Macronutrient restriction may even explain some of the effects previously attributed to caloric restriction. Adult flies that were fed on calorie-diluted diets increased their lifespan relative to those fed on standard diets [21]. However, when the calorie-restricted diets were supplemented with only essential amino acids, this eliminated the increase in lifespan previously observed [21]. Further studies have shown that the ratio of protein to carbohydrate (P:C ratio) in the adult diet is the principle determinant of lifespan in several insects, including *D. melanogaster* and the Queensland fruit fly, *Bactrocera tryoni* [17][22][23]. A more recent study from the same group (2014) shows that dietary in *Bactrocera tryoni* P:C ratios have a major contribution to body composition and fecundity. These studies provide strong experimental evidence that caloric restriction and macronutrient restriction differ in their effects on important life history traits.

The current theory upholds that specific components of the diet may act as signals to trigger a response warning the organism of a nutritionally-stressed state leading to changes in metabolism, physiology, developmental programs, and/or lifespan [15][24]. In humans, macronutrient restriction leads to increased cardiovascular performance and immune function, although additional negative effects have also been reported suggesting trade-offs between life history traits [25]. Further evidence for trade-offs come from studies in flies and beetles showing that the P:C ratios that maximize fecundity differ significantly from those that maximize lifespan [17][23][26]. Presumably, these trade-offs exist to maximize the well-being of the animal under adverse conditions.

In practical terms, one of the most useful tools to simultaneously explore the effects of caloric restriction and macronutrient restriction on life history traits is the Geometric Framework for Nutrition, or nutritional geometry, developed by Steve J. Simpson and David Raubenheimer in the early 90's. This method co-varies the caloric and macronutrient content of the diet across a broad range of values to generate a nutrient space. Nutritional geometry has proven to be a valuable tool for understanding how nutrition affects life history traits across a broad range of species.

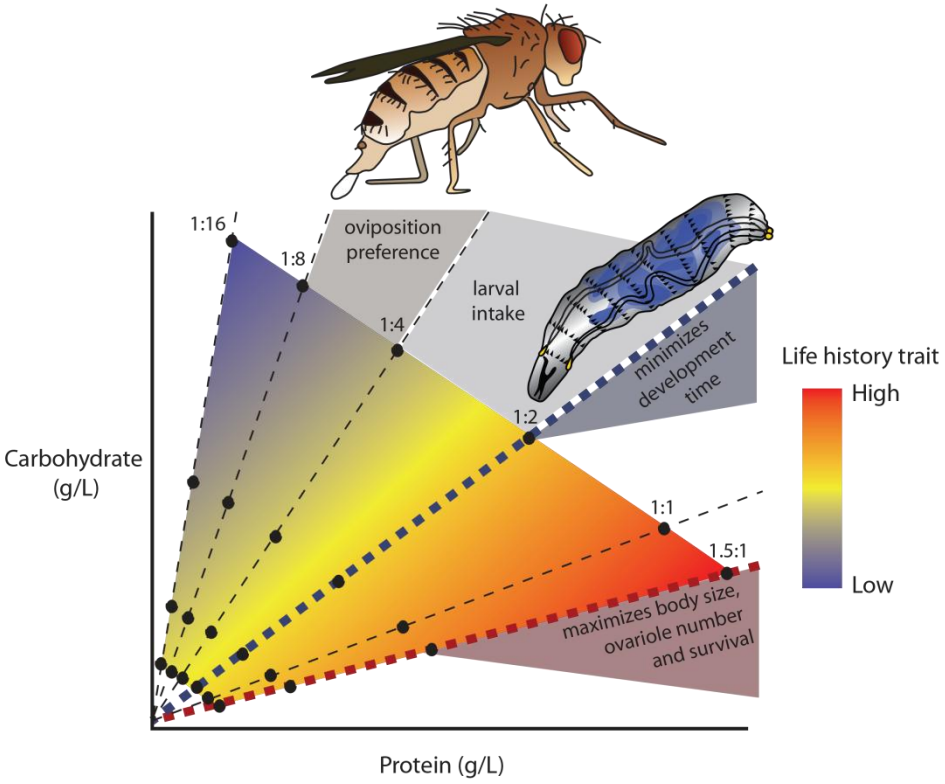

Figure 1.1 – Diagram of a Nutrition Geometry Model, showing how protein and carbohydrates affect life history traits and foraging behavior of Drosophila melanogaster larvae and adult females (Rodrigues, et al, 2005)

1.2 - Mechanisms underlying feeding response in *Drosophila melanogaster* – phenotypic plasticity and resource allocation

Dietary preferences and requirements are expected to play an important role in determining an animal's nutrient intake, allowing them to adjust and compensate for stage-specific nutrient requirements. Responses to the nutritional environment are often heterogeneous, contributing to population-level variation in behavior. Further, many species, including all type of eukaryotes, show changes in a variety of life history traits in response to nutritional cues [21]. Fluctuations in available food resources will maintain a diverse range of responses to these conditions, because different phenotypes will be favored at different times and places. Species of *Drosophila*, *D. melanogaster* included, adapt quickly to caloric restriction within the context of laboratory experimental evolution, and this adaptation bears consequences on a number of life history traits including body size, developmental timing, stress resistance, and longevity [8][11][27][28]. Thus, given sufficient genetic variation for these traits, nutritional requirements can respond to selection and has microevolutionary potential.

Even within a species, animals can show genetic variation that contributes to different degrees of plasticity, leading them to respond differently to food availability, which can represent a constraint that will affect finding or consuming the food [29]. Evidence in *D. melanogaster* suggests that food deprivation early in life predicts deprivation at later stages, known as the chronic adversity scenario. Thus, selection should act to favor a plastic response that produces an adult phenotype that performs well in harsh conditions when larvae encounter nutritional scarcity at critical periods during development [30]. These results suggest that *D. melanogaster* has apparently evolved a variety of different physiological mechanisms to deal with and survive changing environmental conditions, otherwise known as phenotypic plasticity. Mair et al, 2003 suggests that caloric restriction in *D. melanogaster* adults leads to a plastic, adaptive response that induces a reversible alteration in the animal's biology by slowing the accumulation of aging-related damage, adjusting their physiology, metabolism, growth rate, and fertility to account for these altered conditions.

One important way that animals deal with differences in the nutritional environment is the differential allocation of the resources consumed between tissues and organ systems. In insects, nutrient acquisition and allocation may take place in different life stages and the analysis of nutrient routing, via stable isotope tracking, provides a powerful way to understand how the allocation of resources might affect life histories [31]. Constraints on how nutrients can be used may arise from changes in diet, anatomy, digestive physiology, or metabolism across life stages [32]. For example, most of the carbon from sucrose consumed in larval stages appears to be fairly "replaceable" in the development of both larvae and adults, but there is some exceptions concerning the reproductive system. Studies in the fruit fly, tracking resource allocation using isotopic carbon in the larval and adult diets, show that sucrose obtained in larval stages provided ~40% of total somatic carbon in adult females, providing an extremely important energy source for egg provisioning [31]. Work like this reveals the importance of "replaceable" and "nonreplaceable" resources: "nonreplaceable" nutrients are those acquired in the larval stages that are used for the somatic maintenance of the adult, whereas the "replaceable" nutrients can be acquired from either larval or adult dietary sources. Nutrients like sucrose are acquired in larval stages and end up being irreplaceable for the maintenance of adult functions. In some Holometabolous insects, such as *Drosophila melanogaster* or the butterfly *Speyeria mormonia,* aspects of larval nutrition may prove to be limiting or "non-renewable" in adult life [31][33]. These may arise from biochemical constraints on nutrient synthesis, but may be due to diet handling or digestive constraints that make nutrients less accessible to the adult stage [31].

Although the aim of the project is not to understand how particular macronutrients are differentially allocated under different types of juvenile nutritional stress but primarily to understand how plastic responses differ between different nutritional environments, this would make an interesting follow up since metabolic changes can be one of the drivers for these of plastic responses.

1.3 – Nutrition and its impact on life history traits

Core parameters of an animal's life history, such as adult body and organ size and developmental time, are almost uniquely determined in the larval stages. Other traits, like reproductive output, are similarly affected by the larval environment, although variation in adult environment also plays a role. A multitude of environmental conditions affect life history traits during larval development, including photoperiod, temperature, and nutrition [34]. In *D. melanogaster*, the effects of larval nutrition on traits like body and organ size and developmental timing has received the most attention [35][7]. Body mass gained during larval development, which depends on the nutritional composition of the food, determines adult body size since growth stops at the onset of metamorphosis [36]. Reduced developmental nutrition decreases growth rate and reduces final body size.

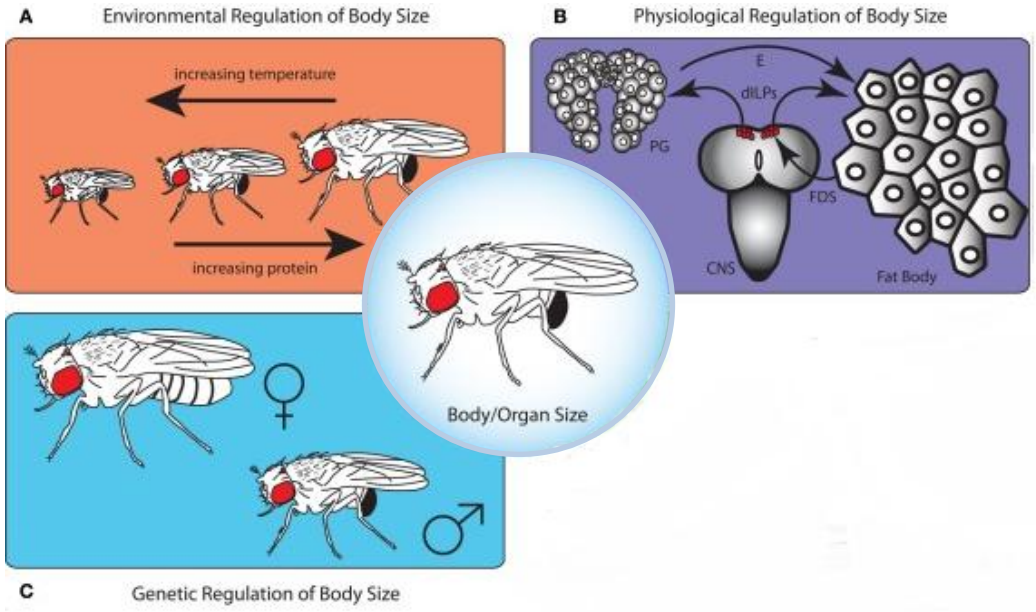

Figure 1.2 –**A)** With increasing temperature, body size tends to decrease. On the contrary, when protein content in larval food increases, adult size increases. **B)** After sensing the diet content, the fat body signals to the central nervous system via a range of secreted peptides to produce and secrete *Drosophila* insulin like petides (dILPs) that will regulate growth and metabolism **C)** The dimorphism in size between females and males is genetically determined in standard conditions. (Adapted from Mirth and Shingleton, 2012)

Protein is a key macronutrient in the larval diet for many life history traits. Increasing protein content in the *D. melanogaster* diet increases body size and the conversation of irreplaceable nutrients, such as protein, in tissues and organs will lead to increased or decreased body size depending on the availability of these resources [34]. But recent studies show that not all larval traits show the same relationship with protein. At intermediate P:C ratios in the larval diet, developmental time is minimized, whereas survival from egg to pupa, male and female body size, and ovariole number is maximized at the highest P:C ratios in *D. melanogaster* [6]. By exploring the effects of caloric and macronutrient restriction in the larval diet, my project fills important gaps in our understanding of the effects of larval diet on life history traits throughout the lifetime of the animal.

1.4 – Effects of nutrition on lifespan and somatic maintenance

Changes in nutrition alter developmental and physiological processes to generate a plastic response in life history traits, including somatic maintenance and survivability. Under favorable conditions, individuals might opt to invest in reproduction at the cost of somatic maintenance and survival; while in under less favorable conditions or stressful conditions they may switch to a state of improved resistance to stress to optimize their fitness [11][37]. Adaptive life history switches may have been shaped by the same selection principle: the evolution of adaptive regulatory plasticity in somatic maintenance and survival. Larval diapause in *C. elegans* [38] or the ovarian arrest in adults' phase of *D. melanogaster* [39] are all examples of how environment changes the rate of somatic maintenance.

Reproduction and lifespan are differently affected by larval versus adult resource availability, and it is unknown how the nutrients acquired in larval or adult state are differentially allocated to somatic and reproductive function. Reproduction is thought to be energetically costly. Further, because both macronutrient and caloric restriction promote longevity at the expense of fecundity, it has been suggested that the longevity–reproduction trade-off represents an energetic resource allocation trade-off in *Drosophila*.

1.5 – Project Aims

My aim with this project was to build a phenotypic dataset to assess the differences in larval and adult life history traits when larvae were reared under different types of

nutritional stress: 1) caloric restriction, which varied the caloric content of the diet but maintained constant the balance of protein and carbohydrates, and 2) macronutrient restriction, which varied the protein to carbohydrate ratio in the diet maintaining constant caloric content. The comparison between the two treatments enables me to understand the role of the macronutrient protein, coupled with or without caloric restriction, in shaping life history traits. These data allow me to assess how the nutritional biology of the larva shapes its development and metabolism to alter traits in both the larva and the adult.

2. Material and Methods

2.1 – Fly population

I used an outbred population of *D. melanogaster*, established in the laboratory in 2007 from 160 Wolbachia-infected fertilized females, caught in the Azeitão, Portugal [40]. The flies were kept in laboratory cages at high effective population size (>1,500 individuals), for 128 non-overlapping generations. Flies were maintained under constant temperature (25°C), humidity (60–70%), and under a 12:12 light-dark cycle, and fed *ad libitum* with standard media containing 45 g of molasses, 75 g of sucrose, 70 g of cornmeal, 20 g of yeast extract, 10 g of agar, 1100 ml of water, and 25 ml of a 10% Nipagin solution per liter of fly food.

Approximately 1000 flies were collected and used to amplify the population over two generations prior to the start of the experiment.

2.2 – Experimental Diets

All diets used in these experiments were based on the IGC Fly Facility standard food recipe (see below), which contains a protein to carbohydrate ratio of 1:10 and a caloric concentration of 1.44 kcal/ml. In this study, I subjected larvae to two types of food treatments: macronutrient dilution and caloric dilution. For the macronutrient dilution experiments, I generated four protein to carbohydrate (P:C) ratios (1:25, 1:50, 1:100 and 1:200), decreasing the amount of protein and increasing the amount of carbohydrates while keeping the calories constant between diets. The four different P:C ratios were isocaloric, since both protein and carbohydrate yield similar amounts of calories per unit mass (1.44 kcal/ml). For the caloric dilution experiments, I made four food types of different caloric values (0.72, 0.36, 0.18 and 0.09 kcal/ml) by diluting the food with 1%

agar to 50%, 25%, 12.5% and 6.25% the concentration of the standard food, maintaining the P:C ratio constant (for the components of each diet, see Table 1). The control food was our standard fly food recipe (details above). For all the diets types, cornmeal and yeast extract were used as protein sources, and cornmeal, yeast extract, sugar and molasses were used as sources of carbohydrates. All pre-weighed dry ingredients were dissolved in sterile distilled water (250 ml) and stirred for 5-10 min. To set the medium, 2 g of agar were added to the suspension before autoclaving it for, approximately, 50 min. To prevent bacterial and fungal growth, we added 0.25% Nipagen and 0.6% (v/v) propionic acid to the cooled mixtures before pouring them into bottles. The bottles were stored in a cold chamber at 4ºC and new medium was made every two weeks.

2.3- Life history traits assays

Approximately 150 flies (100 females and 50 males) were transferred into egg laying chambers (100 ml plastic cups) where they were given an *ad libitum* supply of oviposition substrate (30% agar, 30% sucrose, and 40% apple juice in 60 mm Petri dishes) seeded with live yeast paste for 4 hours. From these dishes, I either separated 30 eggs onto small squares of sterilized paper and randomly distributed each paper square between fly vials containing 7mL of one of the nine diets, or separated 50 eggs into Petri dishes that contained 10 mL of diet, depending on the purpose of the assay. Vials and Petri dishes were maintained at 25 °C in a climate-controlled room under 60–70% humidity.

From the animals reared in each of the diets, I measured several life history traits such as survival from egg to pupa, larval development time from egg to pupa, L2 and L3 growth rate, L2 and L3 mouth hook length, adult wing, femur, thorax, and maxillary palp size, lifespan, and early fecundity. Each assay was replicated ten times.

2.3.1- Egg to pupae survival

The survival from egg to pupa was assessed for the 9 different ratios (4 caloric dilutions, 4 macronutrient dilutions, and the standard food), with 10 replicates for each diet with 30 eggs in each vial. I counted the number of individuals, from the 30 eggs, that initiated metamorphosis (formed prepupa). Pupal eclosion rate was not assessed, and this assay was conducted in parallel with the developmental time assay.

2.3.2 – Egg to pupa developmental time

Using a similar design to the survival study described above, 10 different diets each with 10 replicates/30 eggs per replicate, I assayed the interval of time from egg until pupariation. For this, I counted the number of eggs that hatched in each treatment, the number of white pupa that formed from the hatched eggs, and the time it took for them to initiate metamorphosis on each diet. I checked the vials three times a day (10:00, 14:00 and 18:00) until all the larvae had pupariated or died.

2.3.3. – Duration of the second and third instar larval stages

To stage second instar (L2) and third instar (L3) larvae, I observed them in Petri dishes. In this assay, I used 5 different diets; two caloric restriction (0.36 and 0.09 kcal/ml), two macronutrient restriction (P:C ratios of 1:50 and 1:200), and the standard food as the control. After allowing flies to lay eggs for 4 h, the eggs were separated between the dishes and then I staged larvae twice a day (10:00 and 18:00), maintaining them under constant temperature (25°C) and humidity (60–70%). From day 2, ten individuals were randomly selected from each Petri dish and their developmental stage was recorded using the morphology of the anterior spiracles, before being returned to the Petri dish.

2.3.4 - Dissection of larval mouth hooks

I dissected the mouth hooks from L2 and L3 larvae reared in one of 5 different diets outlined in the previous section. I replicated each treatment 4 times, with 50 eggs per replicate. I collected 10 larvae of each stage, from all the 4 replicates and they were dissected in SH solution with 70% ethanol and 30% glycerol, using a Leica M125 high-resolution stereoscope. The mouth hooks were mounted in a 100% glycerol solution and images were acquired on Zeiss Stereo LUMAR stereoscope, equipped with a Hamamatsu Orca-ER CCD camera and GFP fluorescence filterset, controlled with the MicroManager v1.14 software. The length of the mouth hooks, from the tip of the tooth to the top of the H-piece (Figure 2.1), was measured using the ImageJ software.

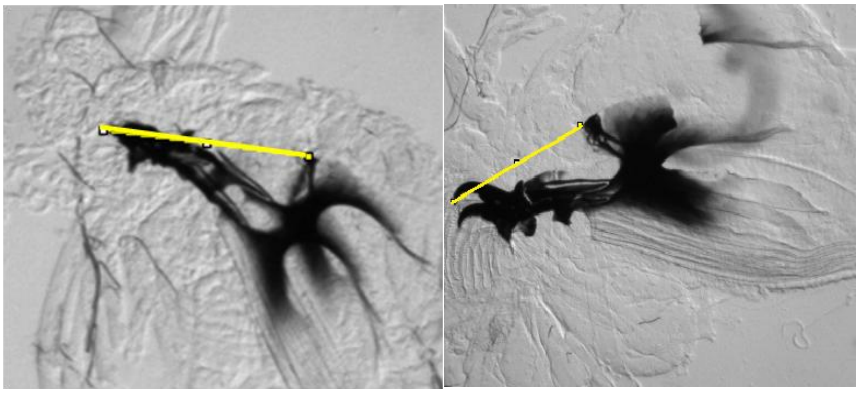

Figure 2.1: Images of larval mouth hooks of *D. melanogaster,* reared in control diet, illustrating the measurements made for all the diets. Mouth hooks from a 2nd instar larva is shown on the left and a 3rd instar on the right. Both pictures were taken with 120x amplification. Morphological measurements of the length are outlined in yellow.

2.3.5- Measurements of adult body parts

I next explored whether macronutrient restriction versus caloric restriction of the diet would differentially affect the size of the adult organs. I dissected adults of all the food treatments and compared the size of the wings, maxillary palps, femur, and thorax. I replicated each treatment 4 times, dissecting 10 flies/replicate (40 flies/treatment). Fly vials were inspected daily at 10:00 and any flies that had eclosed in the previous 24 h were collected and preserved in 80% ethanol. The dissections were made in the SH solution using a Leica MZ75 scope. The different organs were mounted in 100% glycerol solution. Digital images of the wings, maxillary palps, and femur were captured using Zeiss Stereo LUMAR stereoscope, and to measure thorax length, I took dorsal images with a JVC digital camera mounted on a Leica MZ16 binocular microscope.

All the images were processed using ImageJ software; wing and maxillary palp area was estimated using specific landmarks (including vein and bristle positions indicated in Figure 2.2), and femur and thorax length was measured between two anterior and posterior landmarks (Figure 2.2).

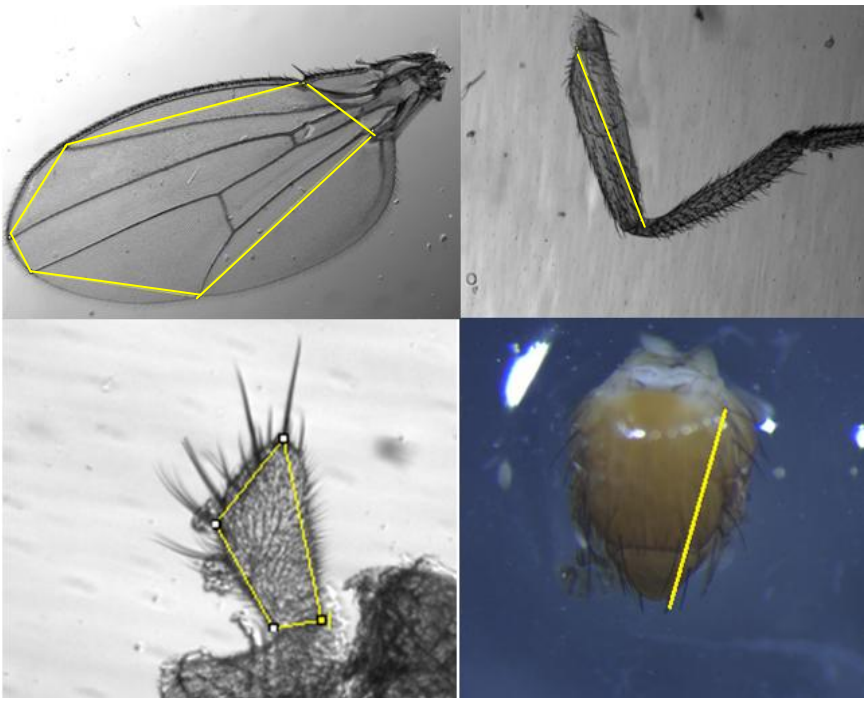

Figure 2.2: Images of *D. melanogaster*, reared in control diet, illustrating the measurements made for all the diets. For each body part, except thoraxes, the organ measured was always from the left side of the body. The femur and maxillary palps were imaged at 120X amplification, wings at 55X amplification, and the thorax was amplified 4.5X. Morphological measurements are outlined in yellow.

2.3.6 – Lifespan and early fecundity

To assess whether the two types of larval diet showed differences in their effects on adult longevity, I designed a lifespan assay for the two caloric dilutions (0.36 and 0.09 kcal/ml), two different P:C ratio (1:50 and 1:200), and the control food. Each treatment was replicated 10 times, with 10 flies per replicate. Since I only used females for this assay, I also looked for the effect of larval diet on early fecundity (1-7 days) of the flies. Adults of both sexes were collected within 24 hours of eclosion from each of the diets, and then transferred into vials containing standard medium. In each vial, I placed 10 males with 20 females, and left them to mate for the first 7 days of the assay. The eggs were counted every day (after eclosing). After the 7 first days, females were separated from the males and transferred to fresh food vials. I followed the lifespan of each adult female until all the flies were dead.

2.3.7 – Statistical analysis

All statistical analyses were conducted using R 3.1.2 software (http://cran.r-project.org/). All measures were plotted over a nutrient array defined by the logarithmic function of the total amount of protein present in each food treatment. The effects on survival from egg to pupa as well as the effects of the caloric restriction treatment on developmental time were fitted using a Gompertz Model. The effects of macronutrient restriction on developmental time and of both dietary treatments on lifespan were analyzed using the Four Parameter Logistic Model. Finally, the effects of dietary treatment on 2nd and 3rd instar duration were fitted using the Asymptotic Regression Model. The analysis of adult body size, larval mouth hook length and early fecundity were fitted using generalized linear models.

These tests were done using the R libraries lme4 (v0.999999, generalized and linear mixed models), coxme (v2.2) and glht (v1.2, multiple comparisons) [1].

Table 1- Protein and carbohydrate content of all 9 diets used in this study.

Treatment	Ratio	Calories (Kcal/mL)	Protein amount per 200 mL food	Carbohydrates amount per 200 mL food
Macronutrient Dilution	1:25	1,44	1,718 g	38,357 g
	1:50	**1,44**	0,85625 g	40,1785 g
	1:100	1,44	0,428125 g	41,08925 g
	1:200	**1,44**	0,21406 g	41,544625 g
Caloric Dilution	1:10	0,72	1,718 g	17,357 g
	1:10	**0,36**	0,85625 g	8,678 g
	1:10	0,18	0,428125 g	4,338 g
	1:10	**0,09**	0,21406 g	2,168 g
Control Food	1:10	1,44	3,425 g	34,714 g

3. Results

3.1 – Survival from Egg to Pupa

I fit the relationship between survival and the log-transformed protein concentration in the diets using the Gompertz Equation, a three parameter non-linear regression model commonly used for fitting survival data (Equation 3.1).

$$y = a \exp(b \exp(cx))$$

Equation 3.1 – Gompertz Sigmoid Function

This model allows me to predict the asymptote of the data (a), the value of y when x is zero (b), and the rate at which y approaches the asymptote – the rate of change in y with x – (c) for each of the diet treatments. I could next ask if fitting the data using the constants specific for each diet type resulted in a better fit than if I assumed all constants were shared between diet types. Significant differences between the model with diet-specific constants versus shared constants meant that the two diets differ in the way that they affect survival. Because in normal food we can assume that survival proportions are close to one, ie. the asymptote should be equal to one, significant differences between diet types are most likely due to differences in the proportion surviving as x approaches zero, or due to difference in the rate of change of survival with protein.

To assess the effects of diet type on survival, I used eight diets that varied in their caloric content, or their macronutrient content. I reared larvae on either four macronutrient restriction diets, including 1:25, 1:50, 1.100, and 1:200, or four caloric restriction diets, 0.72Kcal/ml, 0,36Kcal/ml, 0.18Kcal/ml and 0.09Kcal/ml. For both diet types, survival increased with the increased protein content of the food (Figure 3.1).

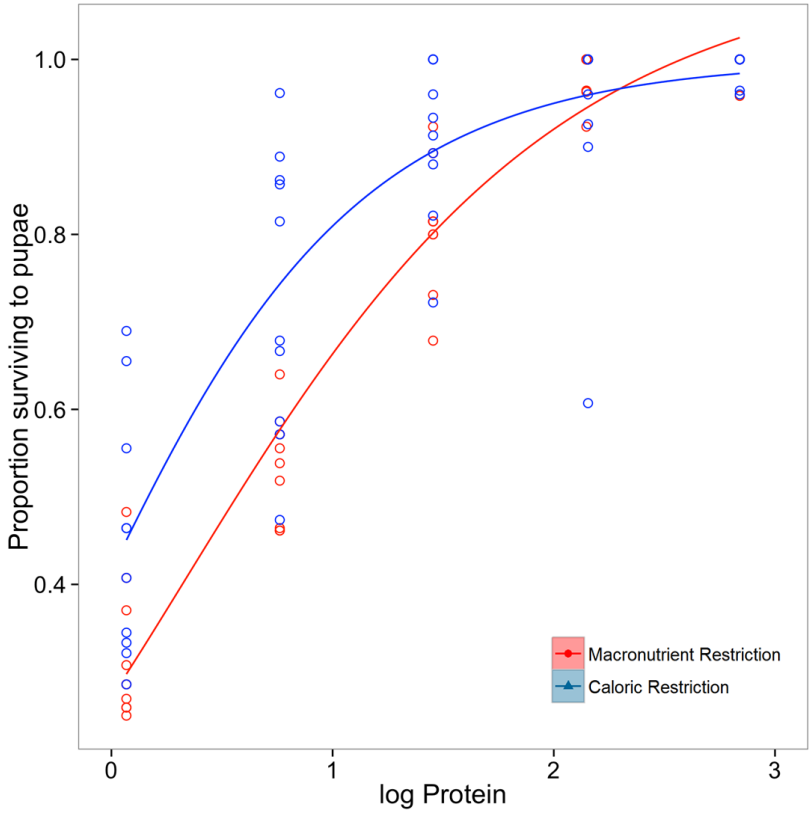

Figure 3.1 – Effects of the different diets in survival from egg to pupa in *Drosophila melanogaster* larvae. The data was fit using the Gompertz model. Survival was measured in standard food **(log Protein=2.98 g)**, in one of four macronutrient restriction diets, either 1:25 (**log Protein= 2.098 g),** 1:50 **(log Protein=1.68 g),** 1:100 **(log Protein=0.68 g)** or 1:200 **(log Protein=0.098 g)**; or in one of four caloric restriction diets, either 50% **(log Protein= 2.098)** , 25% **(log Protein=1.68 g);** 12,5 **(log Protein=0.68 g)** and 6.25% **(log Protein= 0.098 g)**.

Both the predicted constants of the Gompertz function (Table 2) and an ANOVA testing for a significant difference in fit using diet-specific constants versus shared constants (Table 3) indicated that changing the protein content of the food by macronutient restriction has significantly different effects on survival than changing the protein content of the diet by caloric restriction (ANOVA F-value: 11.80, p-value <0.001). For foods of lower protein concentration, macronutrient restriction reduced survival to a greater degree than caloric restriction, resulting in a significant difference in b between diets (Table 3). Neither the asymptote nor the rate of change in proportion surviving with protein differed significantly between diet types (Table 3).

Table 2– Estimates for the diet-specific constants of the Gompertz Sigmoid Function fitting the proportion of larvae surviving to pupae.

	Parameters	Estimate	Std. Error	t- value	*p* - value
Macronutrient Restriction	**a**	1.11119	0.04112	27.02	***
	b	-1.41086	0.06626	21.29	***
	c	-1.00637	0.10605	9.49	***
Caloric Restriction	**a**	0.99888	0.03950	25.285	***
	b	-0.87693	0.09438	9.291	***
	c	-1.42871	0.31424	4.547	***

Table 3 –Analysis of Variance Table (ANOVA) from the comparison between the models using diet-specific constants for the Gompertz function versus shared constants. The estimated values from the ANOVA shows there is a significant difference in the fit to the data when using the diet-specific constants versus a shared constant.

Shared Constant	F-value	*p*-value
a	0.030129	0.0669
b	**18.313**	***
c	2.2831	0.1341
a and b	**10.174**	***
b and c	**13.437**	***
a and c	0.030149	0.1847

3.2 – Egg to pupa developmental time

In this assay we measured the developmental time in the 8 experimental diets, 4 caloric restriction diets and 4 macronutrient restriction diets (as described in the section 2.2 of Material and Methods). The standard diet was included as a control for each diet type. In this assay I observed the number of larvae that reached white three times per day.

In my first attempt to compare the development time across protein concentrations between the diet types, I compared development time using linear models (Sup. Material, Figure 1). However, development time changes non-linearly with protein concentration resulting in a poor fit.

Unable to fit the results with a linear model, I next attempted to fit the data using the same non-linear model, either Gompertz or logistic models. However, it was not

possible to fit the relationship between developmental time and protein from the macronutrient restriction and caloric restriction. I found that the macronutrient restriction could be fit using a four parameter logistic model (Table 4). In my case, I find this model to be reasonable if considering the data to represent the response in developmental time to the dose of protein present in the food. In this function A, B, C and D represent constants that provide the shape of the curve. A is the minimum asymptote; B is the maximum asymptote; C is the mid-point between both asymptotes and D is the rate constant.

$$y = A+(B-A)/1+\exp((C-x)/D))$$

Equation 3.2 – Four Parameter Logistic Function

Development time in response to protein concentration from the caloric restriction diets was best fit using a Gompertz equation, as described above (Table. 5). If the data cannot be fit using the same model, this is strong indication that the shapes of the response for developmental time to the protein content of the diet differ significantly between diet types.

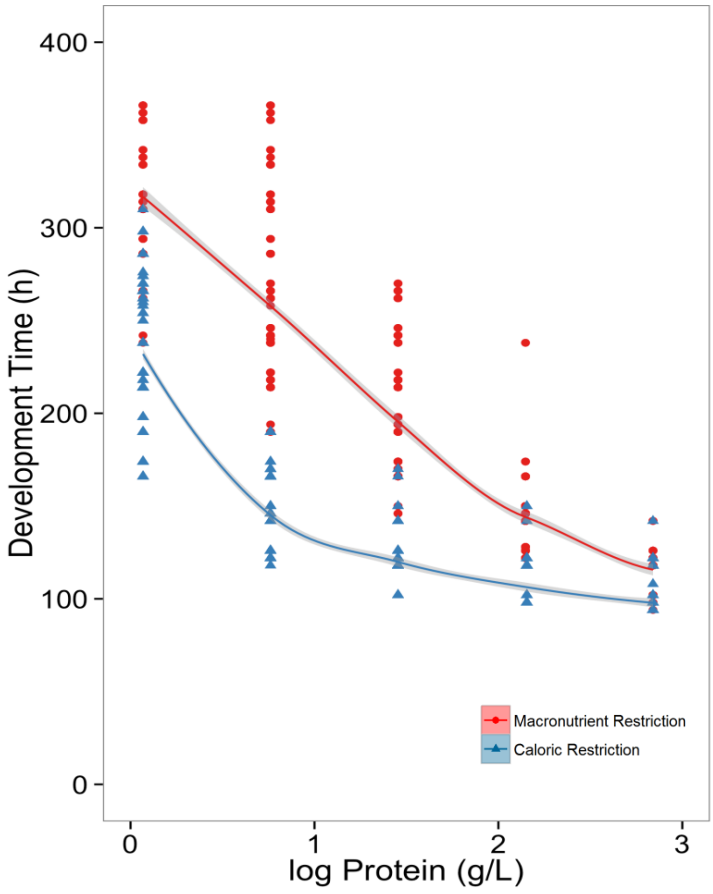

Figure 3.2 – Effects of the different diets in the development from egg to pupa in *Drosophila melanogaster* larvae. Macronutrient restriction was fit using a four parameter logistic growth model. Caloric restriction was fit using the Gompertz model. Developmental time was measured from larvae in standard food **(log Protein=2.98 g)**, in one of four macronutrient restriction, either 1:25 **(log Protein= 2.098 g),** 1:50 **(log Protein=1.68 g),** 1:100 **(log Protein=0.68 g)** or 1:200 **(log Protein=0.098 g)**; or in one of four caloric restriction, either 50% **(log Protein= 2.098)** , 25% **(log Protein=1.68 g);** 12,5 **(log Protein=0.68 g)** and 6.25% **(log Protein= 0.098 g)**.

Table 4 – Results from the analysis of Macronutrient Restriction using the Four Parameter Growth Model.

Parameters	Estimate	Std. Error	t- value	*p* - value
A	5.92282	0.04752	124.65	***
B	0.76141	0.06755	11.27	***
C	1.53293	0.04150	36.94	***
D	4.53852	0.04378	103.66	***

Table 5 – Results from the analysis of Caloric restriction using the Gompertz Sigmoid Function.

Parameters	Estimate	Std. Error	t- value	*p* - value
a	4.523184	0.012499	361.88	***
b	-0.194094	0.002713	71.55	***
c	0.408222	0.014411	28.33	***

3.3 – Duration of the second and third instar larval stages

Next, I explored how long larvae spent in each larval instar relative to diet type. In this assay I choose two diets, from the four original diets of each treatment. The larvae were reared in standard food and in one of two macronutrient restriction diets (1:50 or 1:200) or in one of two caloric dilutions (25% or 6.25%). I focused on the length of the 2nd and 3rd larval instar. Because I could not fit the data from second and third instar with the same model, I used an Asymptotic Regression Model to analyze the change in the proportion of second instars with time and a Four Parameter Logistic Regression to analyze the change in the proportion of third instars with time. The Asymptotic Regression function (Equation 3.3) has three constants labeled Asym, RO and lrc. The first constant gives the upper asymptote, the second is estimated value that intercepts the y-axis and the last one is the natural logarithm of the rate constant, i.e the slope between two separated points in the plot.

$$y\,(t) = \mathrm{Asym} + (\mathrm{RO} - \mathrm{Asym}) * \exp\,(-\exp\,(\mathrm{lrc}) * t)$$

Equation 3.3 – Asymptotic Regression Model Function

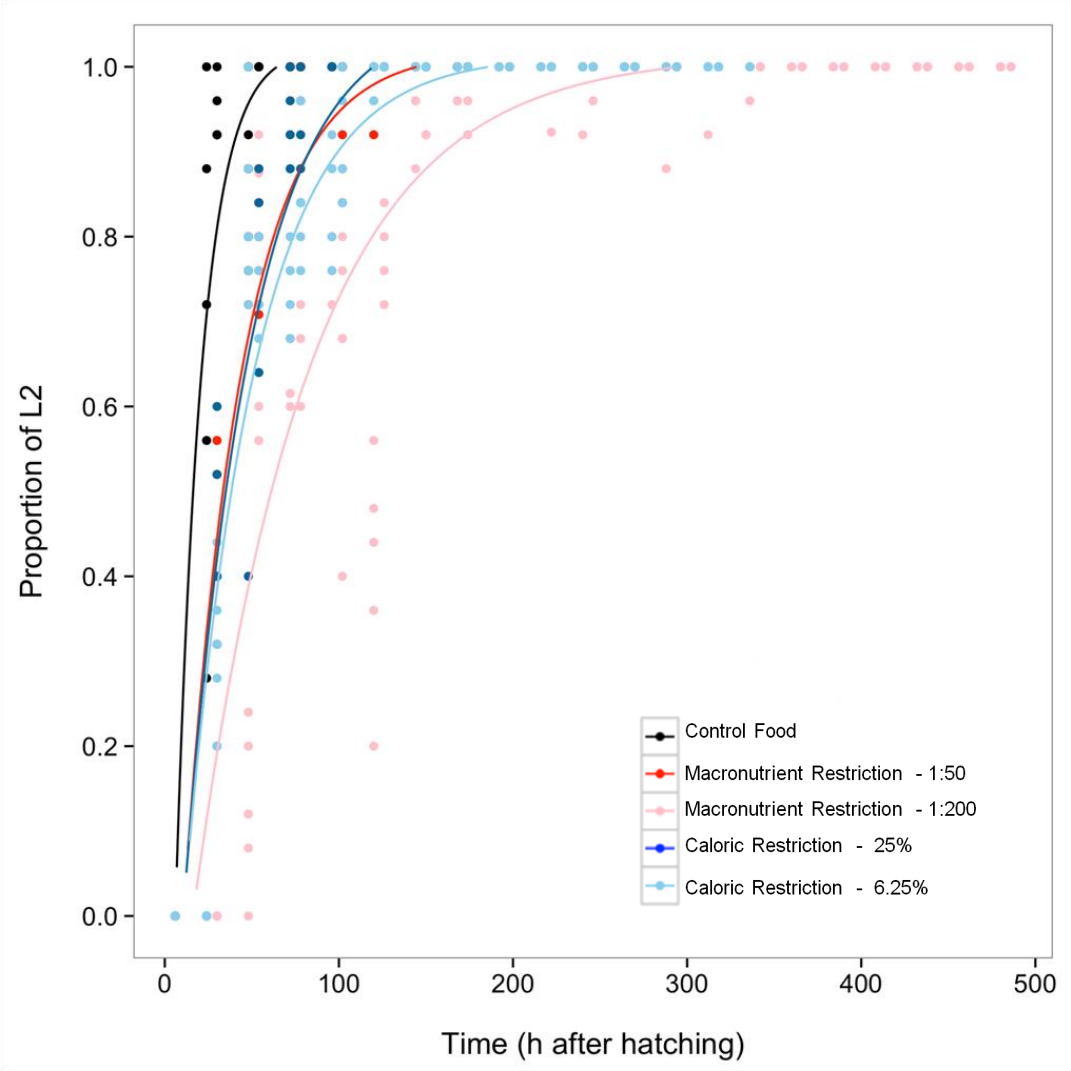

Figure 3.3 – Proportion of second instar larvae, over time in each diet. In this assay larvae were reared in either standard food on one of two macronutrient dilutions, either 1:50 or 1:200, or on one of two caloric dilutions, 25% or 6.25%.

All diet manipulations resulted in a significant delay in the duration of the second instar, when compared to larvae reared in control food (Table 6A). Next we compared the relationship between the proportion of L2 larvae with time between diet types that had equal values of protein, but differed in caloric content (the 1:50 and 25% diets and the 1:200 and 6.25% diets). The diets with intermediate amounts of protein (1:50 ratio and 25% food dilution) do not show significant differences in the proportion of L2 larvae with time (Figure 3.3, Table 6B). On the other hand, diet type affects the duration of the L2 in the diets with the lowest amount of protein (1:200 ratio and 6.25% food dilution), with the 1:200 diet inducing more dramatic delays in L2 duration than the 6.25% diet.

Table 6 – **(A) Comparisons between the duration of second instar in the control diet and the other four diets**. The Asym, RO and lrc parameters are represented as well as the results of F-value. Data was analyzed in R using an Asymptotic Regression Model. The significant differences in the *p*- values are highlighted in bold **(*p<0.05, **p<0.01, ***p<0.001). (B) Comparisons between foods with the same amount of Protein.** Macronutrient Restriction 1:50 diet was compared with the caloric restriction 25% and the Macronutrient Restriction 1:200 was compared with the Caloric Restriction 6,25%.

A)

Treatment	Asymptote	RO	lrc	F-value	*p*-value
Control food	1.02	-0.5	-2.72	/	/
Macro **1:50**	1.02	-0.38	-3.52	34.088	***
Macro **1:200**	1.01	-0.29	-4.19	115.28	***
Caloric **25%**	1.06	-0.33	-3.65	29.398	***
Caloric **6,25%**	1.01	-0.31	-3.70	63.449	***

B)

Comparison between diets of equal protein content	F- value	*p*- value
1:50 vs. **25%**	0.9789	0,4038
1:200 vs. **6.25%**	31.63	***

Table 7 – **(A) Comparisons between the duration of third instar in the control diet and the other four diets**. The constants A, B, C and D are represented as well as the results of F-value. Data was analyzed in R using a Four-Parameter Logistic Model. The significant differences in the *p*- values are highlighted in bold. **(B) Comparisons between foods with the same amount of Protein.** Macronutrient Restriction 1:50 diet was compared with the caloric restriction 25% and 1:200 was compared with the 6,25% diet.

A)

Treatment	A	B	X-mid	Scal	F-value	*p*-value
Control food	-0.016	0.997	35.86	3.46	/	/
Macro **1:50**	-0.0077	0.996	60.68	4.08	325.44	***
Macro**1:200**	-0.001	0.98	112.34	11.65	354.81	***
Caloric **25%**	-4.34E05	0.987	51.19	2.24	90.86	***
Caloric **6,25%**	-0.078	0.99	54.04	12.02	64.008	***

B)

Comparison between diets of equal protein content	F- value	*p*- value
1:50 vs. **25%**	352.04	***
1:200 vs. **6.25%**	165.16	***

Similar to L2 duration, all diets induced significant delays in the duration of the L3 when compared to controls (Figure 3.4, Table 7A). The mid-point between the two asymptotes, defined by constant C, varies between the diets (Table 7B). The diet that shows the most extreme delay is the again the 1:200 diet, with some larvae showing third instar durations of almost 350 hours.

In this instar, larvae reared in the 1:50 ratio and the 25% food reveal significant differences in third instar duration, with the larvae reared in the 1:50 diet spending more time in the third instar. Finally, larvae raised in the lowest protein diets show significant differences in third instar duration, with the 1:200 diet causing longer L3 durations than the 6.25% diet.

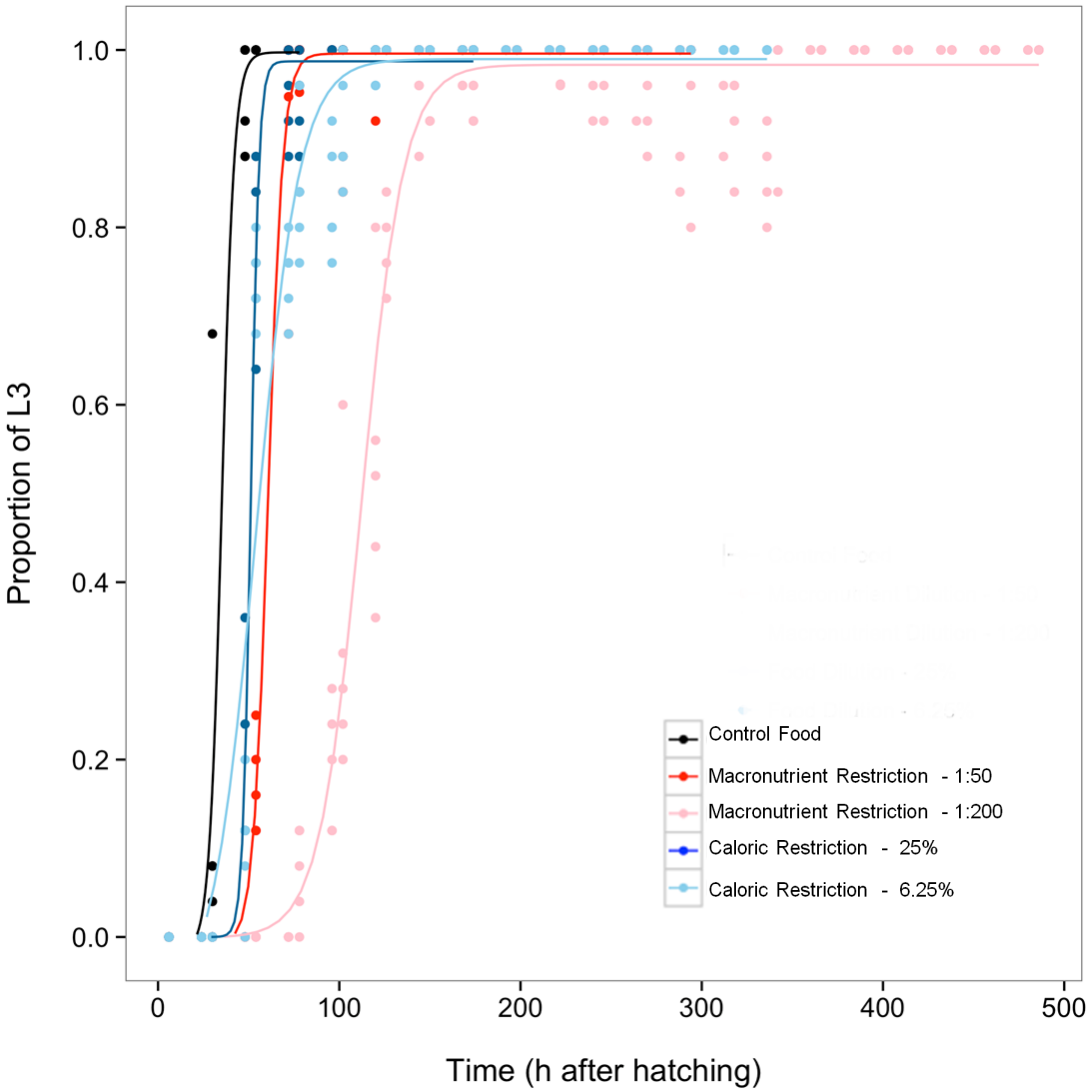

Figure 3.4 – Proportion of third instar larvae, over time in each diet. In this assay larvae were reared in either standard food, on either 1:50 or 1:200, or on either 25% or 6.25% diet.

3.4 – Mouth hooks measurements

Mouth hooks are structures in *Drosophila* larvae that are used to bring food into the mouth by articulating with a chitinized H-shaped posterior sclerite. Before each larval molt, the larvae stop feeding and reduce their movements. At the moult, they expel the mouth hooks from the previous stage, and these are replaced by new mouth hooks from the next larval stage. The size of the mouth hooks is determined by growth in the previous stage. Thus, first instar (L1) mouth hook size reflects embryo size, second instar (L2) mouth hook size reflects growth in the L1, and third instar (L3) mouth hook size reflects growth in the L2. I did not expect there to be significant variation in embryo size between treatments, since we randomly assigned eggs laid by the same population of outbred females to the different feeding treatments. Therefore, as a measure of L1 and L2 larval growth on the different diet types, I measured L2 and L3 mouth hook length.

I observed a clear correlation between the amount of protein in the food and the length of the L2 mouth hooks (Fig 3.5, Table 8A). In addition, the least squared means differed between the two diet treatments, with caloric restriction treatments generating larger mouth hooks at lower protein concentrations than macronutrient restriction (Table 8A). Finally, the interaction term between the log protein concentration and the diet treatment is highly significant. This suggests that reducing protein in the context of the macronutrient dilution differs in its effects than reducing protein via caloric restriction, resulting in a significant difference in the slope of the relationship between L2 mouth hook length and log protein between diet treatments.

In L3 larvae, the mouth hooks showed similar responses to the protein concentration of the diet as it did in L2 larvae (Figure 3.5B, Table 8B). The protein concentration of the diet, the diet treatment, and the interaction between these two variables all had significant effects on the length of the L3 mouth hook. The interaction between the protein concentration and the diet treatment reveals differences in the slopes of both relationships, with a tendency towards larger mouth hook lengths in the low protein concentrations for the caloric restriction treatments.

Figure 3.5 – The effects of macronutrient versus caloric restriction on the relationship between protein concentration and mouth hook length in 2nd instar (A) and 3rd instar larvae (B). Mouth hooks were measured from larvae reared in standard food **(log Protein=2.98 g)**, in one of two macronutrient restriction, either 1:50 **(log Protein=1.68 g)** or 1:200 **(log Protein=0.098 g)**; or in one of two caloric restriction, either 25% **(log Protein=1.68 g)** and 6.25% **(log Protein= 0.098 g)**.

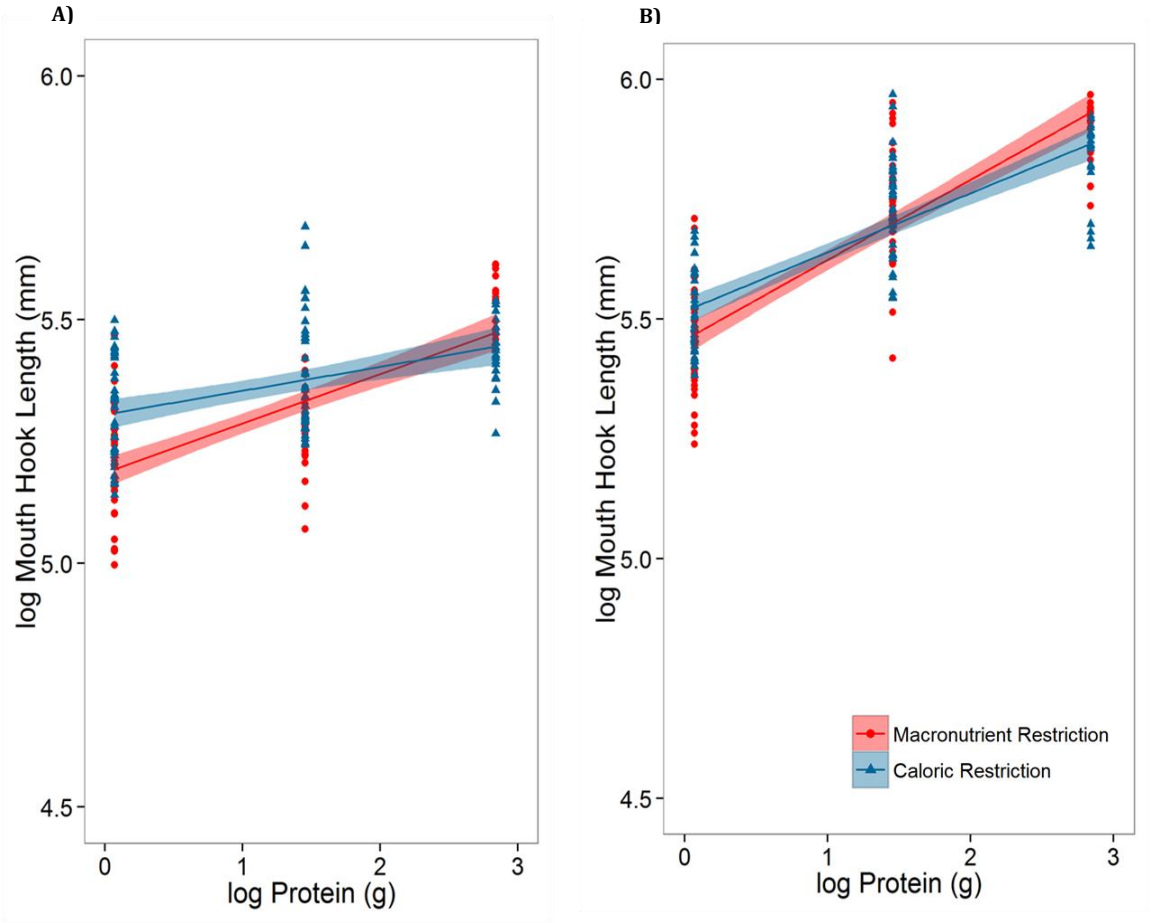

Table 8 – Comparisons in the response of mouth hook length (μm) to protein concentration between the different diet types (macronutrient restriction and caloricrestriction), in 2nd (A) and 3rd instar larvae (B). Data was analyzed in R using Generalized mixed effect models using replicates as the random effect. Significant differences are highlighted in bold.

A)

L2 Mouth Hooks (μm)	Estimates	Pr(>|t|)	*p*-value
LogProtein	0.049079	**5.005**	***
Diet (Macro or Caloric restriction)	0.119312	**5.483**	***
LogProtein : Diet	0.052158	**3.762**	***

B)

L3 Mouth Hooks (μm)	Estimates	Pr(>|t|)	*p*-value
LogProtein	0.123040	**12.874**	***
Diet (Macro or Caloric dilutions)	0.060016	**2.830**	**
LogProtein : Diet	0.043994	**3.255**	**

3.5 – Adult body parts

The results above demonstrate that our two diet treatments induce different effects on the relationship between larval growth and the protein content of the food in the first and second instar. Because adult body and organ size is a product of growth throughout all the larval instars, we next used the measurement of adult traits to estimate the effects of the two diet types on larval growth across all three instars.

In this assay, we reared larvae on either standard diet, one of two macronutrient dilutions (1:50 or 1:200), or one of two caloric dilutions (25% or 6.25%). We then analyzed the size of four adult traits: wing area, maxillary palp area, femur length, and thorax length on males and females that emerged from each treatment.

3.5.1 – Wings

Wing area was measured using 6 landmarks along the veins, as shown in Figure 2.2. The full model, including log protein, diet type, sex, and the interactions between these variables explained 54% of the variation observed in wing area (R^2 adj = 0.54). For both sexes, wing area positively correlated with the protein concentration in the diet (Figure 3.7, Table 9).

Table 9 – Comparisons in the response of wing area (μm^2) between food types. Data was analyzed in R using a Generalized mixed effects model using replicates as the random effects. The significant differences in the *p*-values are highlighted in bold.

Wing Size (μmeter2)	**Chisq**	**Df**	**p-value**
LogProt	158.0650	1	<2.2e-16 ***
Diet (Macro or Caloric dilutions)	0.1360	1	0.712248
Sex	111.5615	1	<2.2e-16 ***
LogProt : Diet	7.4578	1	0.006316 **
LogProt:Sex	0.1826	1	0.669154
Diet :Sex	0.3333	1	0.563693
LogProt: Diet :Sex	0.3424	1	0.558454

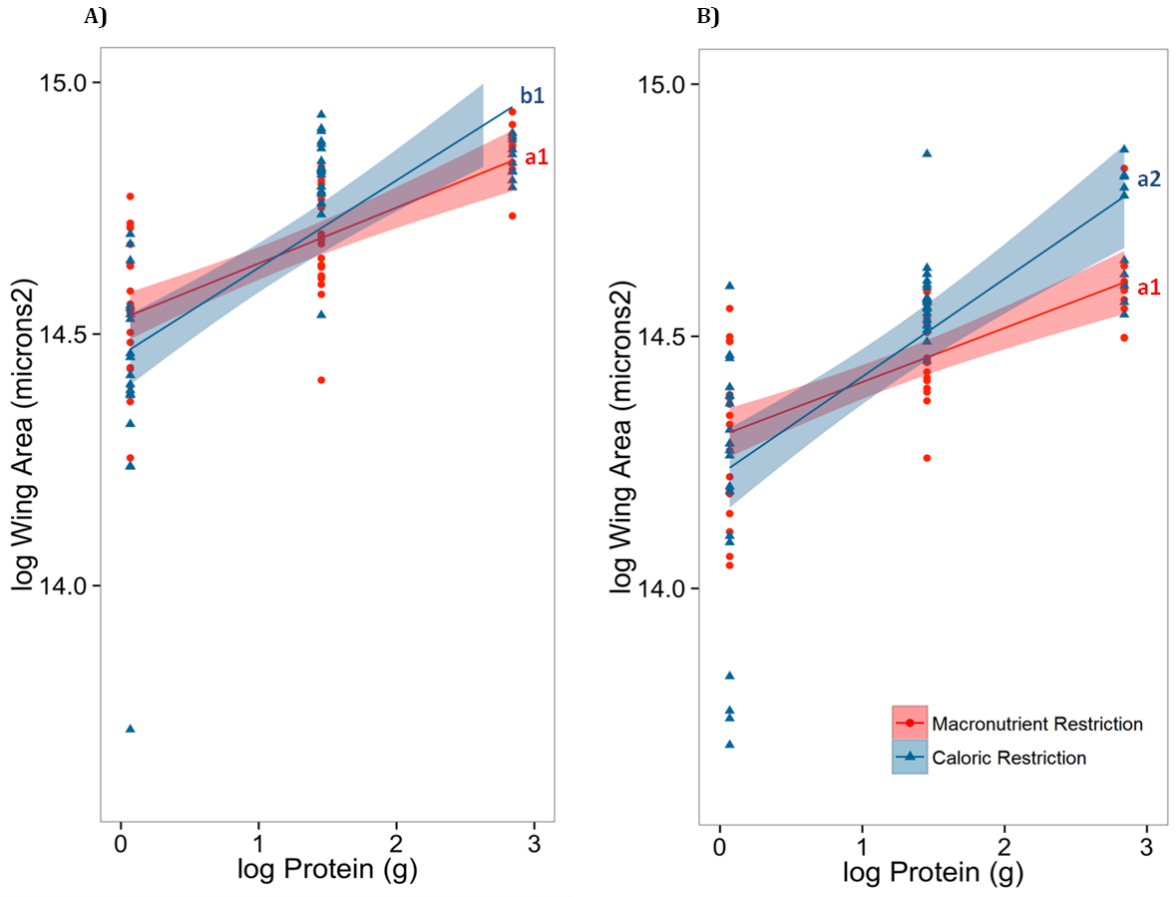

Figure 3.7 – The relationship between wing area and protein concentration in both females (A) and males (B). Larvae were reared on either standard food **(log Protein= 2.98 g)**, on one of two macronutrient restriction, either 1:50 **(log Protein=1.68 g)** or 1:200 **(log Protein=0.098 g)**, or on one of two caloric restriction, either 25% **(log Protein=1.68 g)** or 6.25% **(log Protein= 0.098 g)**. The differences between treatment slopes are represented by letters and the differences in the least square means are represented by numbers.

The least squared means for wing area were not significantly different between diet types, although there was a significant effect of sex on wing area (Table 9). In addition, the interaction between the protein concentration in the food and the diet type is significant (Figure 3.7B, Table 9). Protein concentration had a stronger effect on wing area in the caloric dilution diets than in the macronutrient dilution diets, as determined by the differences in the slope of the lines. The differences in effects between sexes were a result of their differences in their mean values, but not in the slope of the relationship between protein and wing area.

3.5.2 – Maxilary Palp

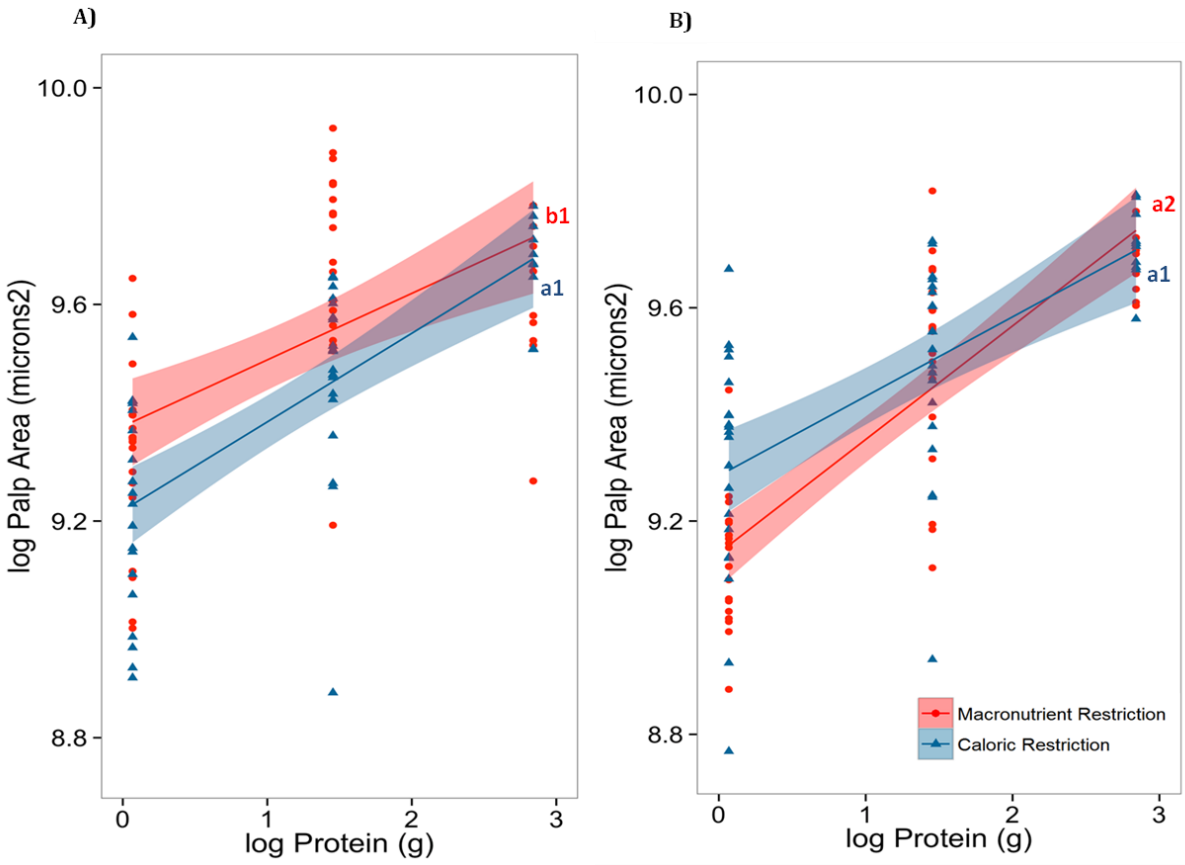

Figure 3.8 – The relationship between maxillary palp area and protein concentration in both females (A) and males (B). These measurements are taken from adults that were reared in, either standard food **(log Protein= 2.98 g)**, on one of two macronutrient restriction, either 1:50 **(log Protein=1.68 g)** or 1:200 **(log Protein=0.098 g)**, or on one of two caloric restriction, either 25% **(log Protein=1.68 g)** or 6.25% **(log Protein= 0.098 g)**. For the differences between treatments, slopes are represented by letters and the differences in the least square means are represented by numbers.

To assess the effects of the dietary treatments on maxillary palp size, I reared larvae in one of the five dietary treatments outlined above, and measured maxillary palp area from the emerging adults using four bristles along the palp as landmarks (Figure 2.2). The full model, including log protein, diet type, sex, and the interactions between these variables explained only 7.4% (R^2 adj= 0.074) of the observed variation in maxillary palp. For both males and females, the size of the maxillary palp showed a significant positive correlation with the protein content of the food. The interaction between the diet type and protein was not significantly different, although there was a significant interaction between sex and diet type. Taken together, these data suggest that although mean

maxillary palp area varied with protein and diet type, the slope of this relationship remained the same for both dietary treatments.

Table 10 – Comparisons in the response of maxillary palp area (mm2) between the different food types. Data was analyzed in R using a Generalized linear model with replicates as the random effect. The significant differences in the *p*- values are highlighted in bold.

Maxilary Palp (μmeter2)	**Chisq**	**Df**	**Pr(>Chisq)**
LogProtein	40.9127	1	1.592e-10 ***
Diet (Macro or Caloric restriction)	0.0119	1	0.91313
Sex	0.1535	1	0.69526
LogProtein : Diet	1.7232	1	0.18928
LogProtein:Sex	0.1553	1	0.69352
Diet :Sex	5.6402	1	0.01755 *
LogProtein:Diet :Sex	1.2770	1	0.25846

3.5.3 – Femur

I measured femur length in the emerging adults using two landmarks (indicated in Figure 2.2). To standardize the data to the previous measurement of the wing and palp, I squared femur length to obtain an estimate of femur area.

The full model, using protein concentration, diet type, sex, and the interaction between all variables, explained 46% of the observed variation in femur area. Femur area showed a significant positive correlation with protein concentration for both males and females (Figure 3.7, Table 11). Further, we saw a significant difference between the sexes in femur area, most likely reflecting sexual dimorphism in body size. Although the interaction terms between protein and sex was not significantly different, diet type significantly changed the slope of the relationship between protein concentration and femur area, meaning that the interaction between the protein amount and the treatment is significant. This appears to be due to differences in slopes between diet treatments in females, but not in males (Fig 3.9 B).

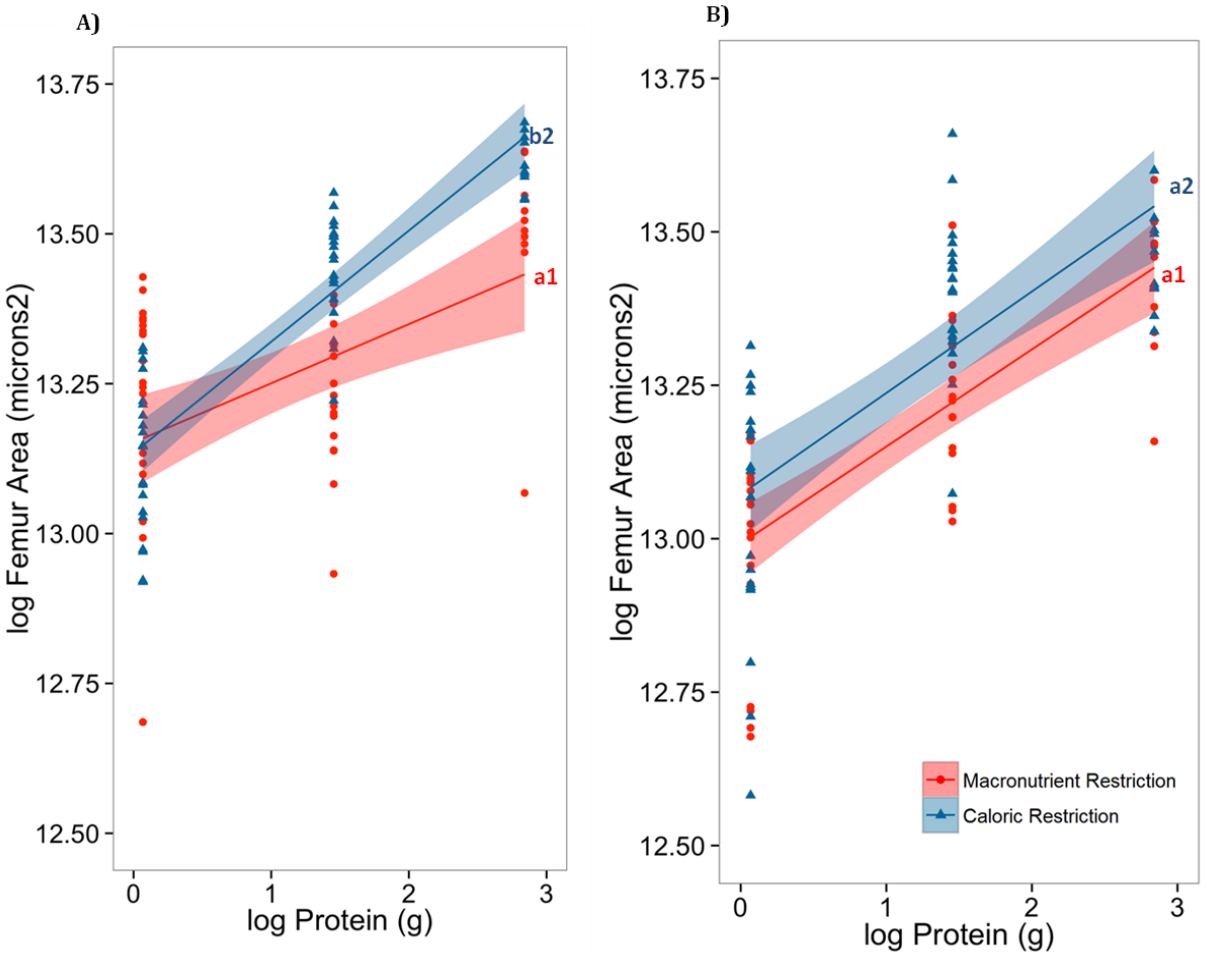

Figure 3.9 – The relationship between femur area and protein concentration in both females (A) and males (B). These measurements are taken from adults that were reared on either standard food **(log Protein= 2.98 g)**, on one of two macronutrient restriction, either 1:50 **(log Protein=1.68 g)** or 1:200 **(log Protein=0.098 g)**, or on one of two caloric restriction , either 25% **(log Protein=1.68 g)** or 6.25% **(log Protein= 0.098 g)**. For the differences between treatments, slopes are represented by letters and the differences in the least square means are represented by numbers.

Table 11 – The relationship between femur area and protein concentration in females (A) and males (B). Data was analyzed in R by a General linear mixed – effects model using replicate as a random effect**.** The significant differences in the *p*- values are highlighted in bold.

Femur Area (μmeter2)	Chisq	Df	Pr(>Chisq)
LogProtein	222.3794	1	2.2e-16 ***
Diet (Macro or Caloric restrcition)	16.7731	1	4.213e-05 ***
Sex	17.3588	1	3.095e-05 ***
LogProtein : Diet	5.2970	1	0.02136 *
LogProtein:Sex	0.9420	1	0.33177
Diet :Sex	0.0085	1	0.92669
LogProtein:Diet:Sex	3.8235	1	0.05054

3.5.4 – Thorax

Finally, I assessed the effects of my two diet treatments on thorax size. I measured thorax length using two bristle landmarks (Figure 2.2). To standardize the thorax dataset to the same dimensions as my previous measures, I took the square of thorax length as an estimate of thorax area.

The full model accounted for 58% of the total variance observed in femur area, with significant effects for protein, sex, and the interaction between protein and diet type. Protein concentration does affect thorax size in females, but there is no significant interaction between protein and diet type. In addition, macronutrient restriction produces stronger reduction in the least squared means for thorax area and has a significantly higher slope than caloric restriction.

Table 12 – **Comparisons in the response of thorax area (μm²) between the different food types**. Data was analyzed in R using a generalized mixed effects model including replicates as the random effect. Significant differences between parameters are highlighted in bold**.**

Thorax Area (μmeter2)	**Chisq**	**Df**	**Pr(>Chisq)**
LogProtein	186.8686	1	<2e-16 ***
Diet (Macro or Caloric restriction)	0.6762	1	0.41090
Sex	163.4658	1	< 2e-16 ***
LogProtein:Diet	5.4889	1	0.01914 *
LogProtein:Gender	0.7188	1	0.39653
Diet:Gender	0.8622	1	0.35311
LogProtein:Diet:Gender	0.2000	1	0.65473

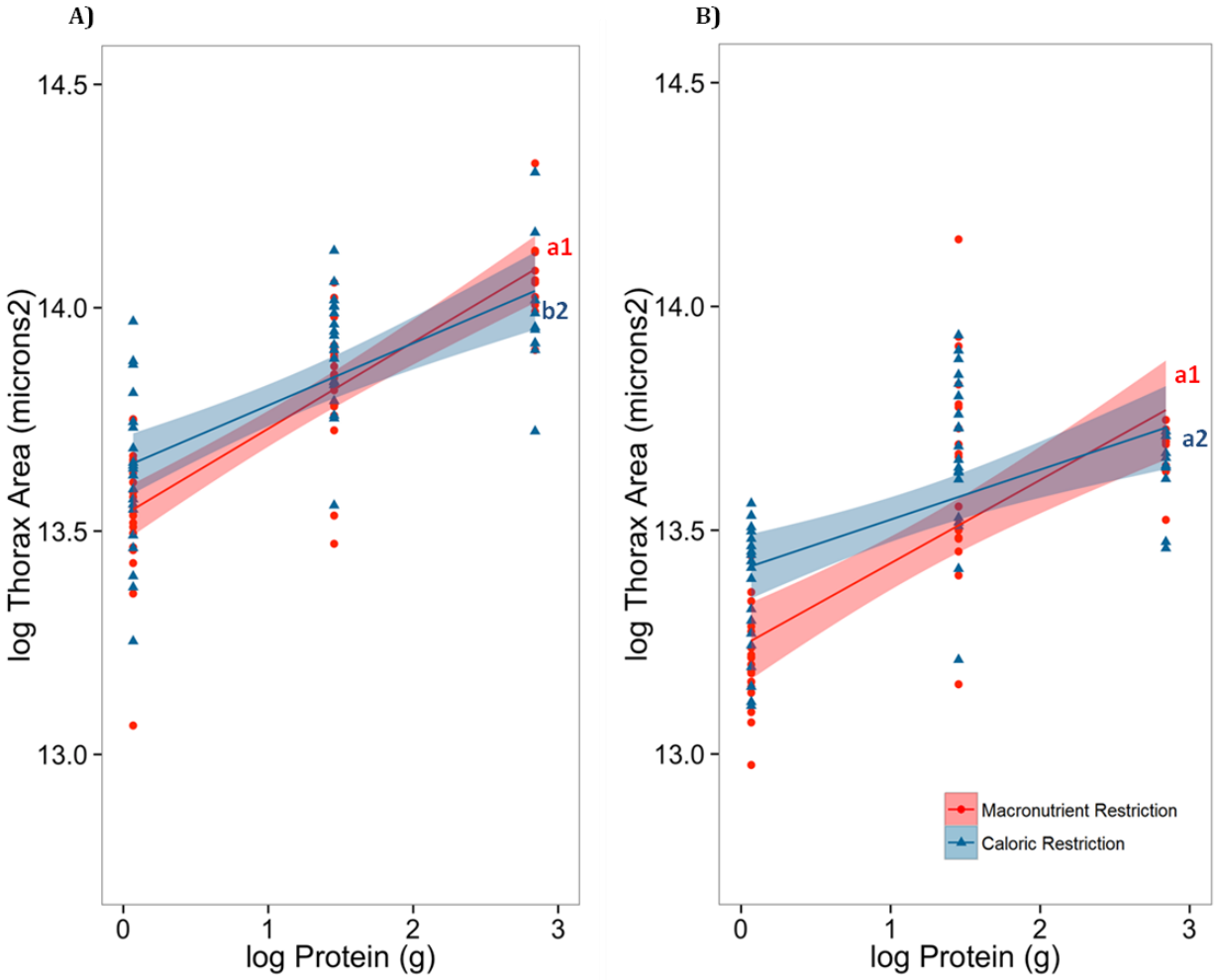

Figure 3.10 – The relationship between thorax area and protein concentration in females (A) and males (B). These measurements are taken from adults that were reared on either standard food **(log Protein= 2.98 g)**, on one of two macronutrient restriction, either 1:50 **(log Protein=1.68 g)** or 1:200 **(log Protein=0.098 g)**, or on one of two caloric restriction, either 25% **(log Protein=1.68 g)** or 6.25% **(log Protein= 0.098 g)**. For the differences between treatments, slopes are represented by letters and the differences in the least square means are represented by numbers.

3.6 – Lifespan

The lifespan assay data was analyzed using a four-parameter logistic model, as described previously. The pattern of survivorship curves varied significantly depending on the diet type. For lifespan, adults emerging from the control food showed intermediate lifespan. Adults exposed to macronutrient restriction as larvae showed the shortest lifespans and those emerging from larvae reared under caloric restriction were the longest lived.

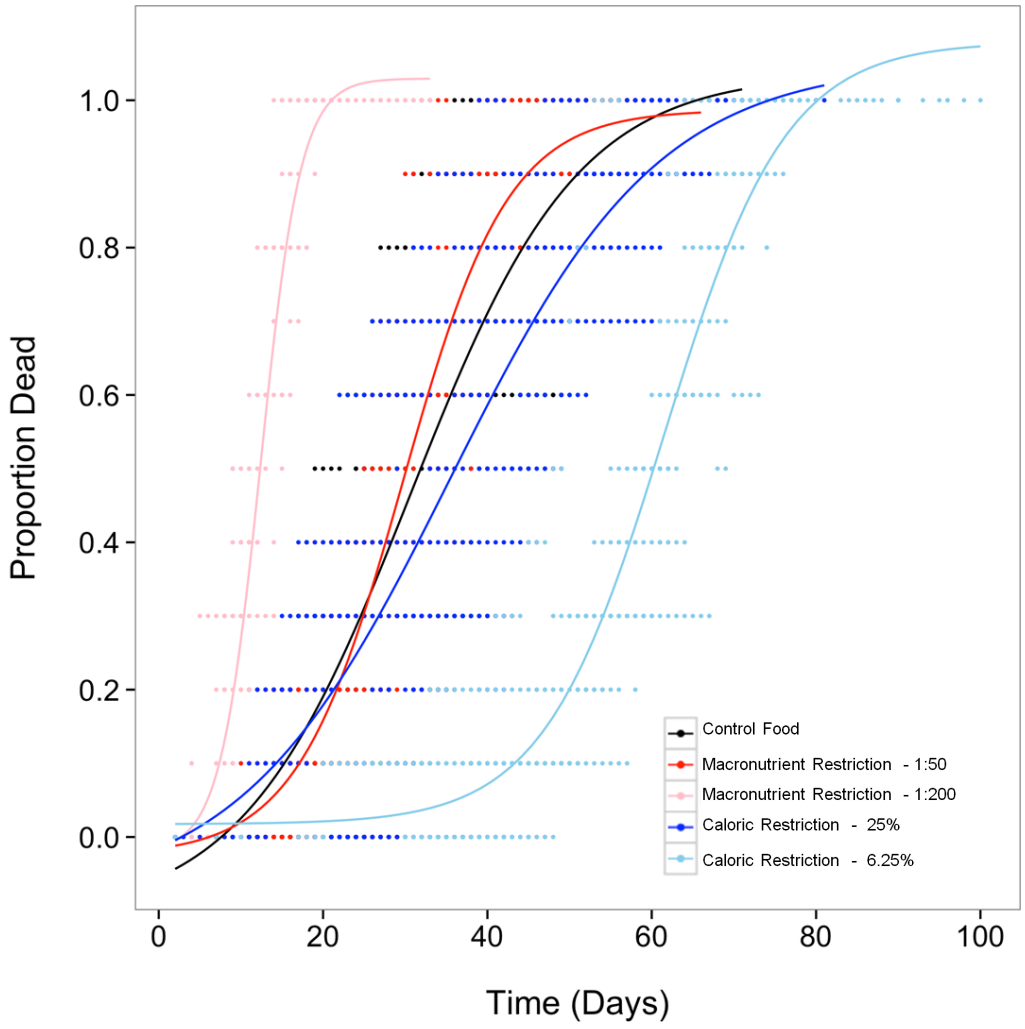

Figure 3.11 – Longevity of *Drosophila melanogaster* adults as a function of larval diet type. In this assay the larvae were reared in either standard food, on one of two macronutrient restriction, either 1:50 or 1:200, or on one of two caloric restriction, either 25% or 6.25%.

Each of the diets was statistically different from control diet for lifespan (Table 13A). We next compared diets with the same protein concentration from the different diet types, the 1:50 and 25% diets and the 1:200 and 6.25% diets. Survivorship patterns of flies reared on the 6.25% diet and the 1:200 ratio showed the greatest difference in lifespan, despite having the same concentration of protein. Flies reared in the 1:200 diet reached the midpoint between the upper and lower asymptotes at 12 days and those reared in the 6.25% diets reached the midpoint at 62 days (Table 13, A and B). Flies reared in the 1:50 and the 25% diets also differed significantly in lifespan, although the difference between treatments was not as dramatic (Table 13B).

Table 13 – **(A) Comparisons of adult longevity in between larval diet types**. The constants A, B, C and D are represented as well as the results of F-value. Data was analyzed in R using a Four-Parameter Logistic Model. The significant differences in the *p*- values are highlighted in bold. **(B) Comparisons between foods with the same amount of protein.**

A)

Treatment	A	B	C	D	F-value	*p*-value
Control food	-0.11	1.04	30.58	10.19	/	/
Macro **1:50**	-0.025	0.99	29.67	6.42	6.618	***
Macro**1:200**	-0.019	1.029	12.38	2.44	7.0446	***
Caloric **25%**	-0.081	1.05	35.41	12.72	10.71	***
Caloric **6,25%**	0.017	1.079	61.57	7.38	7.38	***

B)

Comparison of treatments	F- value	*p*- value
1:50 vs. **25%**	38.377	***
1:200 vs. **6.25%**	784.54	***

3.7 – Early Fecundity

To assess the effects of larval diet type on early fecundity, I put twenty females, whose larvae were reared in one of the four diets tested, and let them lay eggs during seven consecutive days. Every day, the vial was replaced and the eggs were counted. I tested for differences in early fecundity by diet type by fitting the data using a generalized linear model. Because we had no *apriori* assumptions about the shapes of the relationship between the number of eggs laid over time, we fit the data with loess splines and compared the least squared means between treatments. Larvae reared in the control food gave rise to adult females that laid the highest number of eggs in the first days after eclosion (Figure 3.12).

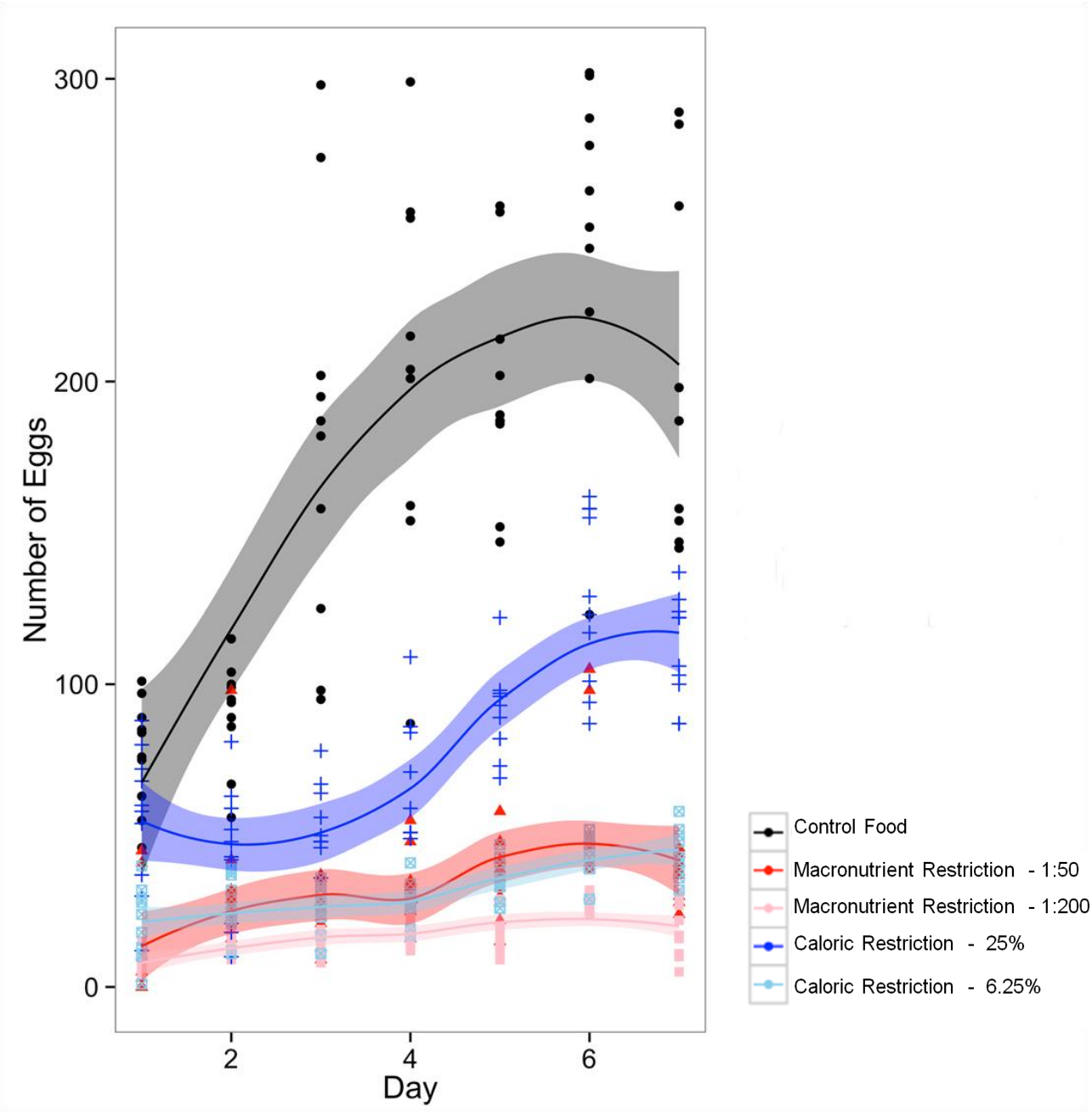

Figure 3.12 – Total number of eggs laid by *Drosophila melanogaster* females in the first seven days after eclosion by larval diet type. The adults that performed these assays were adults whose larvae were reared in either 1:50, 1:200, 25% or 6.25% diets.

Females reared on the 25% caloric restriction diet showed a significant reduction in the number of eggs laid when compare to control, but laid significantly more eggs than all other treatments. Females reared on the 1:50 macronutrient restriction diet and the 6.25% caloric restriction diet laid the same number of eggs, despite the 6.25% diet containing only half the protein content of the 1:50 diet. Finally, the females that laid the fewest eggs were those reared on the 1:200 macronutrient restriction diet (Figure 3.12; Table 14).

Table 14 – A) Results from the General Linear Model comparisons from the eggs laid. The least square means (lsmeans) from each treatment are highlighted in bold. Lsmeans represent the differences in the number of eggs laid in the five diets by the sum of the mean of all the replicates for each diet. **B) The results were grouped, by the model, in four groups (1, 2,3 and 4) meaning that two or more treatments that have similar results are attributed with the same number.** The highest group number represents the highest lsmean and the lowest represents the lowest lsmean value.

A)

Early Fecundity (1- 7 day)	**Chisq**	**D.f.**	***p*- value**
Day	165.62	1	***
Diet (Macro or Caloric dilutions)	1316.10	4	***
Day : Diet	115.86	4	***

B)

Treatment	**lsmean**	**St. Error**	**D. f.**	**Lower. CL**	**Upper. CL**	**Group**
Macro **1:200**	**17.04286**	3.842575	63.74	6.840093	27.24562	1
Macro **1:50**	**33.51429**	3.842575	63.74	23.311521	43.71705	2
Caloric **6,25%**	**32.21429**	3.842575	63.74	22.011521	42.41705	2
Caloric **25%**	**78.24286**	3.842575	63.74	68.040093	88.44562	3
Control Food	**169.85714**	3.842575	63.74	159.6543780	180.05991	4

4 - Discussion

Life histories—describing essential patterns of organismal growth, maturation, reproduction, and survival—show tremendous variation across individuals, species and environments. In this study, I explored how the nutritional composition of the larval diet influences developmental processes and life history traits by manipulating the larval diet

using two types of dietary variation, macronutrient and caloric restriction, and measuring the response of both larval and adult traits. These two treatments induced different responses in both larval and adult performance revealing that, at least for the life history traits assessed, although the quantity of macronutrients in the diet matters, macronutrient balance, particularly protein and carbohydrate, in the larval diet dramatically alters both larval and adult traits.

4.1 – Caloric and macronutrient restriction show differences in their effects on survival from egg to pupa

Organisms carefully regulate macronutrient intake to maximize life history traits. However, when the nutritional conditions are not ideal, they balance the ingestion of specific nutrients that allows them to survive larval development. My data demonstrates that both the protein and the carbohydrate content of the larval diet play an important role in survival from egg to pupae. Survival was maximal in the standard food and decreased in the diets containing less protein. For the same amount of protein, the proportion of eggs surviving to pupae was generally higher in larvae from caloric restriction than from macronutrient restriction. This suggests that for *D. melanogaster,* reducing the protein content of the diet by substituting it with carbohydrate causes greater reductions in survival from egg to pupa than reducing its caloric content.

The fruit fly, and other invertebrates, relies on insulin/IGF signaling (IIS) in order to adapt their metabolic homeostasis, growth or reproduction, to the constant changes in the environment. In *Drosophila,* IIS is stimulated by several peptides called *Drosophila* insulin like peptides (Dilps), which each activate a unique membrane receptor and its downstream signaling cascade [41]. A subset of these Dilps are produced in the central nervous system, in the insulin-producing cells (IPCs), which release ILPs into the hemolymph and act systemically [41]. Dilp secretion is sensitive to the amino acid concentration of the larval diet [42]. The amount of Dilp secreted affects IIS levels during larval development, thereby regulating metabolic homeostasis and growth [34]. Previous work from Géminard and colleagues has shown that deprivation of amino acid in the diet, *Drosophila* larvae experience growth inhibition largely due to a control of Dilp secretion by the IPCs. Amino acid deprivation through a negative feedback mechanism, leads to a decrease in Dilp secretion causing them to be accumulated in the IPC cells. With prolonged exposure of the larvae to this regime, this reduction can have an impact in the carbohydrate metabolism leading to metabolic disorders [41].

My data suggest that reducing protein via caloric restriction versus macronutrient restriction may differentially affect IIS. In general, larvae fed on the diets with the least protein showed reduced survival, however macronutrient restriction reduced survival rates to a greater degree that caloric restriction. We know that decreased protein in the diet leads to a decrease in the IIS, however how this response differs with carbohydrate and caloric concentration in the diet is still unanswered. Work from Pasco and Leopold showed that larvae reared in high sugar/high calorie diets are more prone to accumulate higher levels of circulating glucose and the Adipokinetic hormone (AKH), a glucagon-related hormone. Data from our lab shows that larvae regulate their protein intake more tightly than carbohydrate intake (Carvalho and Mirth, *in preparation*), by ingesting greater volumes of food on low protein diets. This increase in ingestion on the macronutrient dilution diets would result in an increased ingestion of sugar, which may in turn dysregulate larval physiology.

The magnitude of the effects of the diet composition on this specific life history trait can also depend on it genetic context. Since we used an outbred population with high genetic variability, the susceptibility to develop metabolic syndromes related with excess sugar consumption would be expected to vary across genotypes. With this population, it would even be possible to develop a selection experiment to explore the role of genetic variation in susceptibility to metabolic syndromes.

Protein is fundamental for larval growth and development [43], and protein and carbohydrate are the primary source of calories for *Drosophila.* Nevertheless, my experimental design does not account of the contribution and interaction of the other components, such as lipids and vitamins. To distinguish the effects of specific nutrients requires the use a chemically defined diet, such as the holidic diet recently developed by Piper and colleagues (2014) for *D. melanogaster*.

4.2 – Effects of Macronutrient and Caloric Restriction in larval developmental time

4.2.1 - Egg to pupa developmental time

Holometabolous insects have complex life cycles in which nutritional resources acquired during larval feeding are utilized by the pupa and the adult [44]. Indeed, during larval development animals feed intensely to prepare the individual for the non-feeding stage at metamorphosis and for reproductive maturity.

Larvae are vulnerable to predation and faster developmental time is considered advantageous to avoid predation and compete with other larvae [45]. In a study using nutritional geometry, to explore how larval life history traits responded to the protein and carbohydrate composition of the food, showed that *D. melanogaster* larvae minimized developmental time at intermediate P:C ratios (around 1:2) and not in the highest protein foods (P:C ratios of 1:1 and 1.5:1) [6]. My current work further expanded on these findings by exploring how developmental time responds to more severe caloric and macronutrient restrictions. Here, I found that developmental time was minimized in the standard food (containing a P:C ratio of 1:10) and increased as the protein content of the diet decreased. However, for the same amount of protein, developmental time was generally higher in larvae reared under macronutrient restriction than under caloric restriction. However it is still not clear how these different responses occur.

The acquisition and allocation of resources during larval stage is fundamental to support growth, maintain somatic tissue, and influence reproduction and aging, traits all central to life history evolution [41][44]. Because the larval stage represents a significant fraction of the adult energy budget in some insects [33][46], larvae have to change their feeding behavior in response to the diet to survive late larval, pupal, and early adult non-feeding periods. For example, physiological trade-offs may occur across, not just within, life stages and those mat be driven by differences among traits There is likely to be considerable variability in the allocation response to physiological trade-offs that can be more pronounced under environmental stress [46].

Recent work from Pasco and Leopold (2012) showed that in a high sugar/high calorie diet *D. melanogaster* larvae accumulate high levels of circulating glucose and show severe growth inhibition that manifests not only in larval but also in adult body size. This growth deficit was accompanied with a significant developmental delay of three days. Our data demonstrates that this is true even if the diets are isocaloric. However, in the 1:200 ratio, the highest sugar diet, some individuals showed a 350 hour delay in larval development and were much smaller than the control larvae (data not shown).
A trivial explanation for such delay and growth deficit would be that in conditions of increased sugar diets the animals do not feed properly due to the excess sugar and the shortage of protein. Work from Pasco and Leopold also found that larvae raised on high sugar/high calorie diets showed reduced ingestion rate compared to animals on normal food. Those results also supported work from Musselman and colleagues that saw wild-type wandering third instar larvae raised on either high-sugar, high fat, or high-protein

diets were reduced in size. Although I did not measure the ingestion rate in each, data from our lab show that larvae in low P:C ratios consume greater volumes of food. Thus, I suspect that low levels of ingestion are unlikely in this case.

Previous work showed that larvae reared on a high sugar diets over-produce and release high quantities of Dilps to counteract increased glycemia [41]. More specific larvae quantification of the expression and production levels of the different Dilps genes in the larval brain showed that larvae raised in diets with higher amount of sugars showed a two-fold increase in Dilp peptide accumulation, therefore suggesting an immediate increase of Dilp production in the brain, upon increased sugar ingestion. Results from the same study showed that after a long exposure to high sugar diet, larvae revealed a general reduction of IIS, itself a consequence of Dilp resistance in peripheral tissues. Increase in insulin production and secretion is associated with increased glycemia and is a characteristic of insulin resistance, a phenotype shared with Type 2 Diabetes data from mammalian studies [47].

The evolutionary origins of this "signaling" are still not completely understood but recent studies demonstrate the importance of a nutrient sensitive signaling pathway as a crucial mediator for phenotypic plasticity in *Drosophila melanogaster* larvae in response to the concentration and combination of dietary nutrients.

4.2.2 – Duration of the second and third instar larval stages

Given the overall increase in developmental time in diets with reduced protein, I next sought to assess the effects of caloric and macronutrient restriction on the duration of the second and third instar. Developmental time in both instars was delayed in all the diets except in the standard food. In the first instar larvae, I did not notice significant delays in instar duration (data not shown), however in the second instar all larvae showed significant delays in diets with reduced protein when compared with the standard food. Not surprisingly, dietary composition showed the strongest affects on the duration of the third and final larval stage.

Developmental time is controlled by checkpoints and feedback systems that keep the neuroendocrine system 'informed' about the nutritional state of the organism [10]. These endocrine responses during larval molts establish when the larvae are of sufficient size to enter metamorphosis [48]. The third larval instar is a particularly important stage, since it is when larvae reach the critical weight that corresponds to a developmental switch in the way the larva responds to starvation; after reaching critical weight starvation

no longer delays metamorphosis [49]. As a consequence, the plastic response of adult size to nutrition reflects the growth occurring after the critical size, whereas variation in developmental time mostly reflects the time needed to reach critical size [10].

Several studies have shown that the insulin-dependent growth of the prothoracic glands, the glands that synthesize the molting hormone ecdysone, determines when critical weight is reached in *Drosophila* [49][50]. In the early third instar, ecdysone titers slowly increase, reaching a peak around 9 hours after third instar ecdysis [51]. This peak of ecdysone synthesis depends on dietary protein and induces the developmental transition at critical weight. In unbalanced macronutrient restriction diets, the ability to synthesize the hormone may be further compromised, demonstrating that carbohydrates also have an important effect on ecdysone synthesis at critical weight.

4.3 – Effects of nutrition on the growth of larvae and adult morphological traits

4.3.1 – Second and third instar mouth hooks measurements

Adult size in insects is determined by the final size of the larvae and becomes fixed once the larvae enter metamorphosis [49]. During the last 3 days of larval development, *Drosophila melanogaster* increases 200-fold in mass, with nutrient reserves accumulating [44]. The growth of the larva at various stages can be assessed by larval weight, or estimated by measuring the length of the larval mouth hooks.

I found significant differences in larval growth for both the first and second instar, with a clear correlation between the amount of protein in the food and the length of the L2 and L3 mouth hooks. Macronutrient restriction generated smaller mouth hooks than caloric restriction, with more striking effects in L2 mouth hooks. My data suggest that reducing protein in the context of the macronutrient restriction results in more severe inhibition of growth than in the context of caloric restriction and also that that protein reduction induces different effects on larval stages. Notably, the diets in which larvae develop faster are also the diets in where L2 and L3 larvae have the largest mouth hooks.

4.3.2 – Adult body parts

Within species, the variation in the body size is usually accompanied by the variation in size of its constituent body parts, or traits, a relationship called static allometry [52]. Regarding environmental factors, it is well known that some factors, such as

temperature, nutrition, and rearing density, can contribute to variation for body and organ size in *Drosophila,* but not necessarily in the same manner [52].

Adult body and organ size is a direct product of growth throughout all the larval instars and by measuring adult traits we can estimate the effects of different diets on larval growth across all three instars. As an approximation for the effect of the two diet types on total larval growth, I measured four adult traits: wings, femur, thorax and maxillary palp. By measuring adult organs, I could further characterize the effects of the two diet types on the size of individual organs, to test if all organs respond in the same way to the diets.

Several studies have reported that for *Drosophila melanogaster,* increasing protein content of the diet increases adult body [34]. The factors that coordinate organ growth in response to nutrition include circulating Dilps and amino acids [53], which influence growth via the IIS and target of rapamycin (TOR) pathways, respectively. The nutritional plasticity of individual traits in *Drosophila melanogaster* appears to reflect their sensitivity to changes in signaling. Previous work suggested that upon nutritional stress, for example amino acid deprivation, *Drosophila* larvae experience growth inhibition largely due to a reduction in circulating Dilps [41].

I found that these four traits respond differently to the diets with some presenting striking differences across the diets. Macronutrient and caloric restriction diets did not induce the same response in all the traits or even the same response in a given trait between sexes. All traits were largest in the standard food diets. With wing and femur size, I observed greater plasticity in response to protein concentration in the caloric restriction diets than in the macronutrient restriction diets. Males and females also differed in their response and, for example, I only found changes in plasticity for wing and maxillary palp size between diet types in females. My results show that body parts respond differently depending on the diet and sex.

Diets with low protein give rise to smaller individuals, but in addition Pasco and Leopold (2012) showed that larvae reared in high sugar/high calorie diets, which contain the same concentration of protein as the control diets, metamorphose into adults with a smaller body size (-16.9%). Despite elevated circulating Dilp levels in larvae reared on high sugar diets, they showed that the growth deficit observed in high sugar diet is caused by a general reduction of IIS after a consequent Dilp resistance in peripheral tissues in the larvae. Both results show that the effects of protein concentration on body and organ size depends on the carbohydrate context.

4.4 - Lifespan

Diet affects the quality and the duration of life in a wide range of living organisms [18]. McCay published, in 1935, the first paper demonstrating that reduced intake of nutrients without malnutrition, now called caloric restriction, could increase the mean as well as the maximum lifespan of rats. Several studies followed using a diverse range of species, including *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *Drosophila melanogaster*, all suggesting that reducing nutrient intake increased lifespan [1][18].

The fruit fly is now the most used organism for the investigation of the mechanisms by which caloric restriction extends lifespan, essentially due to the ease of genetic manipulations in this animal. More than 40 years ago, David and colleagues reported that longevity of laboratory *D. melanogaster* was extended when flies were fed with diluted diets with different dilutions of yeast, cornmeal, and other carbohydrates. Subsequent studies corroborated these results, further highlighting the effects of caloric restriction on lifespan [1][8][9][11]. In addition, several studies demonstrate that caloric restriction increases resistance to various forms of stress (for example starvation or oxidative stress) and this is one of the reasons why it increases lifespan [54].

Many studies have been done to understand the importance of specific macronutrients in the diet and we know now that is possible to obtain the benefits to lifespan through a suitable balance of nutrients in the diet [9].

In this project, I aimed to understand the effects in adult female lifespan by comparing the effects of caloric and macronutrient restriction in the larval diet. My results showed that adult females raised on caloric restriction diets lived much longer than control flies. This shows that caloric restriction in both the larval and adult diets extend lifespan. However, adults reared in the macronutrient restriction diets as larvae showed dramatically reduced lifespan when compared to those reared on control diets. This is in direct opposition to the effects of macronutrient restriction in the adult diet since feeding adults on diets with low P:C ratios greatly extends lifespan [23]. Further, Partridge and colleagues (2009) manipulated all the components of a defined diet in adults and concluded that replacing protein with either carbohydrates, lipids or vitamins was sufficient to extend lifespan. However adding one essential amino acid alone, methionine, was sufficient to shorten lifespan in females. Importantly, my work highlights that the macronutrient composition of the diet can have strikingly different effects on lifespan depending on developmental stage.

Both larval and adult macronutrient restriction may be regulated by a common molecular mechanism, the insulin/ insulin-like growth factor (IGF) pathway. Adding

essential amino acids to the diet decreased lifespan in normal flies but only slightly decreased the lifespan in flies with a mutation in the insulin-like receptor. During development and growth, IIS is known to interact with 'target of rapamycin' (TOR) signaling, a nutrient-sensing pathway and it is tempting to speculate that amino acids might also act through TOR to affect lifespan [55]. The TOR pathway appears to sense nutrients cell autonomously in the fat body of the larva and then act non-cell autonomously to regulate growth by modifying the secretion of insulin-like peptides [42][50].

Even with the very strong protein restriction in the two macronutrient restriction diets with shorter lifespan than control, there was probably an effect of increased amount of sugar that was unbalancing the positive effects of the reduction in protein. Work from Musselman and colleagues (2001) showed that high-calorie diet fed to developing larvae induced phenotypes, in larvae and adults, that were consistent with insulin resistance. The larvae developed severe hyperglycemia, had reduced body size and overexpressed several genes encoding DILPs. Since it is known that increased insulin signaling decreases lifespan [56], flies in the macronutrient restriction diets suffer from this metabolic effect. Also, Pasco and Leopold (2012) showed that feeding larvae a high sugar/ high calorie diet resulted in hyperinsulinemic phenotype that eventually lead to insulin resistance and abnormalities in growth, IIS related. However, how increasing IIS in larvae changes metabolic or physiological responses in adult life is still not completely understood.

4.5 - Early fecundity

Survival and reproduction may be mutually constrained by the competitive allocation of nutrients, and the increased allocation of resources to one function can reduce the pool of metabolites available for the other [60]. This is what is called the "costs of reproduction", a particular kind of trade-off between life history traits when an increase in one life history trait (reproductive trait) that improves fitness is coupled to a decrease in another life history trait (survival) that reduces fitness [11]. Such trade-offs between survival and reproduction can have at least two different sources: on the one hand fecundity might reduce survival because of the costly production of gametes, and on the other hand, survival might decrease due to the elevated mortality risk associated with courtship and mating behavior [37]. Nutrient acquisition is likely to moderate the severity or occurrence of tradeoffs since the quality and quantity of food during pre -adult stages affect adult

lifespan and reproductive capacity in a variety of animals, as predicted by the reallocation hypothesis [61].

Studies in the trade-off between lifespan and egg production show that the macronutrient conditions optimizing lifetime egg production, P:C ratios of 1:4 differed significantly from those that optimized lifespan, P:C ratios 1:32 [23]. Similar results were found in the Queensland fruit fly [22]. Early studies by Chapman and Partridge (1996), varying the yeast and carbohydrate composition of the adult diet, showed that intermediate food concentration produced the greatest median longevity among females.

In *D. melanogaster* resource allocation seems to shift toward somatic function under conditions of restricted nutrition [37]. Several studies have shown that *D. melanogaster* reared in diets with low availability of yeast had a reduced rate of reproduction. Good and Tatar (2001) showed that when fed a diet of sugar and yeast, females produce many eggs and this happens because without yeast, oogenesis is arrested at previtellogenic stages. This reduction in egg production is thought to be due to a reduction in juvenile hormone (JH) synthesis. If JH deficient females are treated with an analog of JH, methoprene, vitellogenesis is restored [62].

Since nutrition affects general growth of the animal, the development of the ovary is reduced in the diets that produce smaller adults [4][6]. This affects the number of eggs females lay. Ovary size is determined in the larval stages, measured by the ovariole number, the egg-producing functional unit of the ovary. Ovariole number correlates with larval nutrition, especially with the protein content of the diet [4][63]. In fact, work done in *D. melanogaster* shows that ovariole number is maximized at high P:C ratios (1.5:1) [4][44]. My data supports these results; larvae reared in food containing the highest amount of protein gave rise to adults that laid the most eggs in the first 7 days after eclosion. Macronutrient restriction affects fecundity more than caloric restriction. Females from larvae reared in the 1:200 diet laid significantly less eggs than females from the 6.25% diet, although the protein contents of the two diets were the same. This may support the idea that very high content of carbohydrates in the larval diet, when combined with low amount of protein, further reduces either ovariole number or egg maturation rate.

5 – Conclusions

In my study, I found that the caloric and macronutrient restriction play important and distinct roles in regulating larval and adult life history traits in *Drosophila melanogaster*. Depending on the diet in which the larvae are reared, these diet types induce clear differences in larval and adult life history traits. With the final goal of understanding stage-specific effects of nutrition in shaping the response of larval and adult life history traits, this project ended up revealing how developmental nutrition can change the overall performance of the fly. I also found that although trait plasticity depends on protein, important interactions between protein and sugar modify both larval and adult phenotypes. Furthermore, my data demonstrates that diet types can induce divergent effects on phenotype depending on the developmental stage. I propose that these differences in larval response to dietary constraints may be the result of a complex metabolic and endocrine responses, lead by the IIS pathway, with consequences on the regulation of other developmental hormones. With knowledge produced in this field we may be one step closer to explaining the classical explanations of reproduction, ageing, and how they interact to generate nutrition-related metabolic syndromes.

Bibliography

[1] **Fontana, L., & Partridge, L.** (2015). Promoting health and longevity through diet: From model organisms to humans. *Cell*, *161*(1), 106–118.

[2] **Scholte, R. S., van den Berg, G. J., & Lindeboom, M.** (2015). Long-run effects of gestation during the dutch hunger winter famine on labor market and hospitalization outcomes. *Journal of Health Economics*, *39*, 17–30.

[3] **Stein, A. D., Pierik, F. H., Verrips, G. H. W., Susser, E. S., & Lumey, L. H.** (2009). Maternal exposure to the Dutch famine before conception and during pregnancy: quality of life and depressive symptoms in adult offspring. *Epidemiology (Cambridge, Mass.)*, *20*(6), 909–915.

[4] **Matavelli, C., Carvalho, M. J. A., Martins, N. E., & Mirth, C. K.** (2015). Differences in larval nutritional requirements and female oviposition preference reflect the order of fruit colonization of Zaprionus indianus and Drosophila simulans. *Journal of Insect Physiology*, *82*, 66–74.

[5] **Simpson, S. J., Le Couteur, D. G., & Raubenheimer, D.** (2015). Putting the balance back in diet. *Cell*, *161*(1), 18–23.

[6] **Rodrigues, M. A., Martins, N. E., Balance, L. F., Broom, L. N., Dias, A. J., Fernandes, A. S., ... Mirth, C. K. (2015)**. Drosophila melanogaster larvae make nutritional choices that minimize developmental time. *J Insect Physiol*, *81*, 69–80.

[7] **Partridge, L., Piper, M. D. W., & Mair, W.** (2005). Dietary restriction in Drosophila. *Mechanisms of Ageing and Development*, *126*(9 SPEC. ISS.), 938–950.

[8] **Flatt, T.** (2009). Ageing: Diet and longevity in the balance. *Nature*, *462*(7276), 989–990.

[9] **Grandison, R. C., Piper, M. D. W., & Partridge, L.** (2009). Amino-acid imbalance explains extension of lifespan by dietary restriction in Drosophila. *Nature*, *462*(7276)

[10] **Kolss, M., Vijendravarma, R. K., Schwaller, G., & Kawecki, T. J.** (2009). Life-history consequences of adaptation to larval nutritional stress in drosophila. *Evolution*, *63*(9), 2389–2401.

[11] **Flatt, T., Heyland A.** (2011). 1st ed, Oxford. Oxford University press.

[12] **Klass, M. R.** (1983). A method for the isolation of longevity mutants in the nematode Caenorhabditis elegans and initial results. *Mechanisms of Ageing and Development*, *22*(3-4), 279–286.

[13] **Hansen, M., Flatt, T., & Aguilaniu, H.** (2013). Reproduction, fat metabolism, and life span: What is the connection? *Cell Metabolism*, *17*(1), 10–19.

[14] **Mair, W., Goymer, P., Pletcher, S. D., Partridge, L., Mair, W., & Partridget, L.** (2003). Demography of Dietawy Restriction and Death in Drosophita. *Science*, *301*(5640), 1731–1733.

[15] **Min, K. J., Flatt, T., Kulaots, I., & Tatar, M.** (2007). Counting calories in Drosophila diet restriction. *Experimental Gerontology*, *42*(3), 247–251.

[16] **Overgaard, J., Tomčala, A., Sørensen, J. G., Holmstrup, M., Krogh, P. H., Šimek, P., & Koštál, V.** (2008). Effects of acclimation temperature on thermal tolerance and membrane phospholipid composition in the fruit fly Drosophila melanogaster. *Journal of Insect Physiology*, *54*(3), 619–629.

[17] **Fanson, B. G., & Taylor, P. W.** (2012). Protein:carbohydrate ratios explain life span patterns found in Queensland fruit fly on diets varying in yeast:sugar ratios. *Age*, *34*(6), 1361–1368.

[18]**Heilbronn, L. K., & Ravussin, E.** (2003). Calorie restriction and aging : review of the literature and implications for studies in humans. *The American Journal of Clinical Nutrition.* 1-3.

[19] **Guarente, L., & Kenyon, C.** (2000). Genetic pathways that regulate ageing in model organisms. *Nature*, *408*(6809), 255–262.

[20] **Merry, B. J.** (2002). Molecular mechanisms linking calorie restriction and longevity. *The International Journal of Biochemistry & Cell Biology*, *34*(11), 1340–1354.

[21] **Carvalho, G. B., Kapahi, P., & Benzer, S.** (2005). Compensatory ingestion upon dietary restriction in Drosophila melanogaster. *Nature Methods*, *2*(11), 813–815.

[22] **Fanson, B. G., Fanson, K. V., & Taylor, P. W.** (2012). Cost of reproduction in the Queensland fruit fly: Y-model versus lethal protein hypothesis. *Proceedings of the Royal Society B: Biological Sciences*, (October), 4893–4900.

[23] **Lee, K. P., Simpson, S. J., Clissold, F. J., Brooks, R., Ballard, J. W. O., Taylor, P. W., … Raubenheimer, D.** (2008). Lifespan and reproduction in Drosophila: New insights from nutritional geometry. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(7), 2498–503.

[24] **Bruce, K. D., Hoxha, S., Carvalho, G. B., Yamada, R., Wang, H. D., Karayan, P., … Ja, W. W.** (2013). High carbohydrate-low protein consumption maximizes Drosophila lifespan. *Experimental Gerontology*, *48*(10), 1129–1135.

[25] **Good, R. A., & Lorenz, E.** (1992). Nutrition and cellular immunity. *International Journal of Immunopharmacology*, *14*(3), 361–366.

[26] **Ja, W. W., Carvalho, G. B., Zid, B. M., Mak, E. M., Brummel, T., & Benzer, S**. (2009). Water- and nutrient-dependent effects of dietary restriction on Drosophila lifespan. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(44), 18633–18637.

[27**] Taormina, G., & Mirisola, M. G.** (2014). Calorie restriction in mammals and simple model organisms. *BioMed Research International*, *2014*.

[28] **Nijhout, H. F., Riddiford, L. M., Mirth, C., Shingleton, A. W., Suzuki, Y., & Callier, V.** (2014). The developmental control of size in insects. *Wiley Interdisciplinary Reviews: Developmental Biology*, *3*(1), 113–134.

[29] **Kaun, K. R., Chakaborty-Chatterjee, M., & Sokolowski, M. B.** (2008). Natural variation in plasticity of glucose homeostasis and food intake. *The Journal of Experimental Biology*, *211*(Pt 19), 3160–6.

[30] **Nettle, D., Frankenhuis, W. E., & Rickard, I. J.** (2013). The evolution of predictive adaptive responses in human life history. *Proc Biol Sci*, *280*(1766), 20131343.

[31] **Min, K. J., Hogan, M. F., Tatar, M., & O'Brien, D. M.** (2006). Resource allocation to reproduction and soma in Drosophila: A stable isotope analysis of carbon from dietary sugar. *Journal of Insect Physiology*, *52*(7), 763–770.

[32] **Min, K. J., & Tatar, M.** (2006). Drosophila diet restriction in practice: Do flies consume fewer nutrients? *Mechanisms of Ageing and Development*, *127*(1), 93–96.

[33] **Boggs, C. L. and Freeman, K. D.** (2005). Larval food limitation in butterflies: effects on adult resource allocation and fitness. *Oecologia* **144**, 353-361

[34] **Mirth, C. K., & Shingleton, A. W.** (2012). Integrating body and organ size in Drosophila: Recent advances and outstanding problems. *Frontiers in Endocrinology*, *3*(APR), 1–13.

[35] **Baldal, E. A., Van Der Linde, K., Van Alphen, J. J. M., Brakefield, P. M., & Zwaan, B. J.** (2005). The effects of larval density on adult life-history traits in three species of Drosophila. *Mechanisms of Ageing and Development*, *126*(3), 407–416.

[36] **Sang, J H.** 1956. The quantitative nutritional requirements of *Drosophila melanogaster*. *J Exp Biol*, 33, 45–72.

[37] **Flatt, T., Amdam, G. V, Kirkwood, T. B., & Omholt, S. W.** (2013). Life-history evolution and the polyphenic regulation of somatic maintenance and survival. *Q Rev Biol*, *88*(3), 185–218.

[38] **Braendle C., Milloz J., Félix M.-A.** (2007). Mechanisms and evolution of environmental responses in *Caenorhabditis elegans*. *Current Topics in Developmental Biology* 80:171–207.

[39] **Tatar M., Yin C.-M.** (2001). Slow aging during insect reproductive diapause: why butterflies, grasshoppers and flies are like worms. *Experimental Gerontology* 36:723–738.

[40]**Martins, N. E., Faria, V. G., Teixeira, L., Magalhães, S., & Sucena, É.** (2013). Host Adaptation Is Contingent upon the Infection Route Taken by Pathogens. *PLoS Pathogens*, *9*(9).

[41] **Pasco, M. Y., & Leopold, P.** (2012). High sugar-induced insulin resistance in Drosophila relies on the Lipocalin Neural Lazarillo. *PLoS ONE*, *7*(5), 1–8.

[42] **Géminard, C., Rulifson, E. J., & Léopold, P.** (2009). Remote Control of Insulin Secretion by Fat Cells in Drosophila. *Cell Metabolism*, *10*(3), 199–207.

[43] **Lett Torsten Kristensen, B. N., Overgaard, J., Loeschcke, V., Mayntz, D., & Kristensen, T. N.** (2010). Drosophila melanogaster fat and viability in Dietary protein content affects evolution for body size, body fat and viability in Drosophila

melanogaster, (October 2010), 269–272.

[44] **Aguila, J. R., Hoshizaki, D. K., & Gibbs, A. G.** (2013). Contribution of larval nutrition to adult reproduction in Drosophila melanogaster. *J Exp Biol*, *216*(Pt 3), 399–406.

[45] **Krijger, C. L., Peters, Y. C., & Sevenster, J. G.** (2010). Competitive Ability of Neotropical Drosophila Predicted from Larval Development Times Competitive ability of neotropical Drosophila predicted from larval development times. *Oikos*, *92*(2), 325–332.

[46] **Boggs, C. L.** (2009). Understanding insect life histories and senescence through a resource allocation lens. *Funct. Ecol.* **23**, 27-37.

[47] **Wang, Y., Lam, K. S. L., Kraegen, E. W., Sweeney, G., Zhang, J., Tso, A. W. K., … Xu, A.** (2007). Lipocalin-2 is an inflammatory marker closely associated with obesity, insulin resistance, and hyperglycemia in humans. *Clinical Chemistry*, *53*(1), 34–41.

[48] **Nijhout, F.** (1975). Dynamics of juvenile hormone action in larvae of the tobacco hornworm, Manduca sexta. *Biology Bulletins*.

[49] **Mirth, C., Truman, J. W., & Riddiford, L. M.** (2005). The role of the prothoracic gland in determining critical weight for metamorphosis in Drosophila melanogaster. *Current Biology*, *15*(20), 1796–1807.

[50] **Colombani, J.** (2005). Antagonistic Actions of Ecdysone and Insulins Determine Final Size in Drosophila. *Science*, *310*(5748), 667–670.

[51] **Koyama, T., Rodrigues, M. a, Athanasiadis, A., Shingleton, A. W., & Mirth, C. K.** (2014). Nutritional control of body size through FoxO-Ultraspiracle mediated ecdysone biosynthesis. *eLife*, *3*(July 2015), 1–20.

[52] **Shingleton, A. W., Estep, C. M., Driscoll, M. V, & Dworkin, I.** (2009). Many ways to be small: different environmental regulators of size generate distinct scaling relationships in Drosophila melanogaster. *Proceedings. Biological Sciences / The Royal Society*, *276*(1667), 2625–2633.

[53] **Edgar, B. a.** (2006). How flies get their size: genetics meets physiology. *Nature Reviews. Genetics*, *7*(12), 907–16.

[54] **Piper, M. D. W., Mair, W., & Partridge, L.** (2005). Counting the calories: the role of specific nutrients in extension of life span by food restriction. *The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences*, *60*(5), 549–555.

[55] **Tatar, M., Post, S., & Yu, K.** (2014). Nutrient control of Drosophila longevity. *Trends in Endocrinology and Metabolism*, *25*(10), 509–517.

[56] **Tatar, M.** (2001). A Mutant Drosophila Insulin Receptor Homolog That Extends Life-Span and Impairs Neuroendocrine Function. *Science*, *292*(5514), 107–110.

[60] **Kirkwood, T. B., & Rose, M. R.** (1991). Evolution of senescence: late survival sacrificed for reproduction. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *332*(1262), 15–24.

[61] **Bergland, A. O., Behrman, E. L., O'Brien, K. R., Schmidt, P. S., & Petrov, D. A.** (2014). Genomic Evidence of Rapid and Stable Adaptive Oscillations over Seasonal Time Scales in Drosophila. *PLoS Genetics*, *10*(11).

[62] **Tu, M., Yin, C., & Tatar, M.** (2002). Impaired ovarian ecdysone synthesis of Drosophila melanogaster insulin receptor mutants. *Aging Cell*, *1*(2), 158–160.

[63] **Mendes, C., Mirth, C.K.** (2016). Stage-specific plasticity in ovary size is regulated by insulin / insulin-like growth factor and ecdysone signalling in Drosophila. *Genetics, 202.*

[64] **Piper, M. D. W., Blanc, E., Leitão-Gonçalves, R., Yang, M., He, X., Linford, N. J., … Partridge, L.** (2014). A holidic medium for Drosophila melanogaster. *Nature Methods*, *11*(1), 100–5.

[65] **Dominiak, B. C., Sundaralingam, S., Jiang, L., Fanson, B. G., Collins, S. R., Banos, C., … Taylor, P. W.** (2014). Evaluating Irradiation Dose for Sterility Induction and Quality Control of Mass-Produced Fruit Fly Bactrocera tryoni (Diptera : Tephritidae). *Journal of Economic Entomology*, *107*(3), 1172–1178.

[66] **Good, T. P., & Tatar, M.** (2001). Age-specific mortality and reproduction respond to adult dietary restriction in Drosophila melanogaster. *Journal of Insect Physiology*, *47*(12), 1467–1473.

[67] **Mair, W., & Dillin, A.** (2008). Aging and survival: the genetics of life span extension by dietary restriction. *Annual Review of Biochemistry*, *77*, 727–754.

[68] **Partridge L, Alic N, Bjedov I, Piper MDW.** (2009) Ageing in *Drosophila*: The role of the insulin/Igf and TOR signalling network. *Experimental Gerontology*. 2011;46(5):376-381.

[69] **Musselman, L. P., Fink, J. L., Narzinski, K., Ramachandran, P. V., Hathiramani, S. S., Cagan, R. L., & Baranski, T. J.** (2011). A high-sugar diet produces obesity and insulin resistance in wild-type Drosophila. *Disease Models & Mechanisms*, *4*(6), 842–849.

[70] **Mair, W., Sgrò, C. M., Johnson, A. P., Chapman, T., & Partridge, L.** (2004). Lifespan extension by dietary restriction in female Drosophila melanogaster is not caused by a reduction in vitellogenesis or ovarian activity. *Experimental Gerontology*, *39*(7), 1011–1019.

Supplementary Information

Table S1 – Standard food composition and content amount per 100g.

Control Food	Protein (100 g)	Carbohydrates (100g)	Amount (g)	Protein Food	Carbohydrates Food
Molasses	4,5	88	45	2,025	39,6
Sugar	0	100	75	0	75
Cornmeal	7	73,7	70	4,9	51,59
Yeast Extract	51	36,9	20	10,2	7,38
Agar	0	0	10	0	0
Total			220	17,125	173,57
				P:C ratio **1:10**	Calories **1.44Kcal/ml**

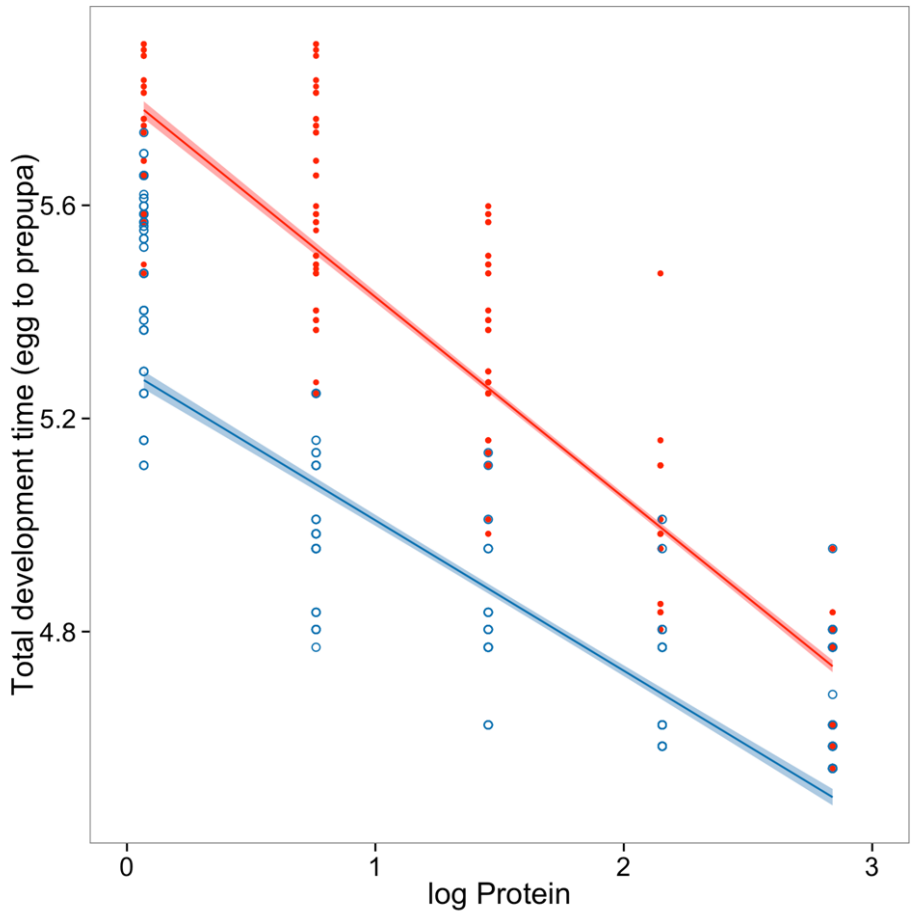

Figure S1 – Effects of the different diets in the development from egg to pupa in *Drosophila melanogaster* larvae. Macronutrient and Caloric restriction treatments were both fit using a General Linear Model.

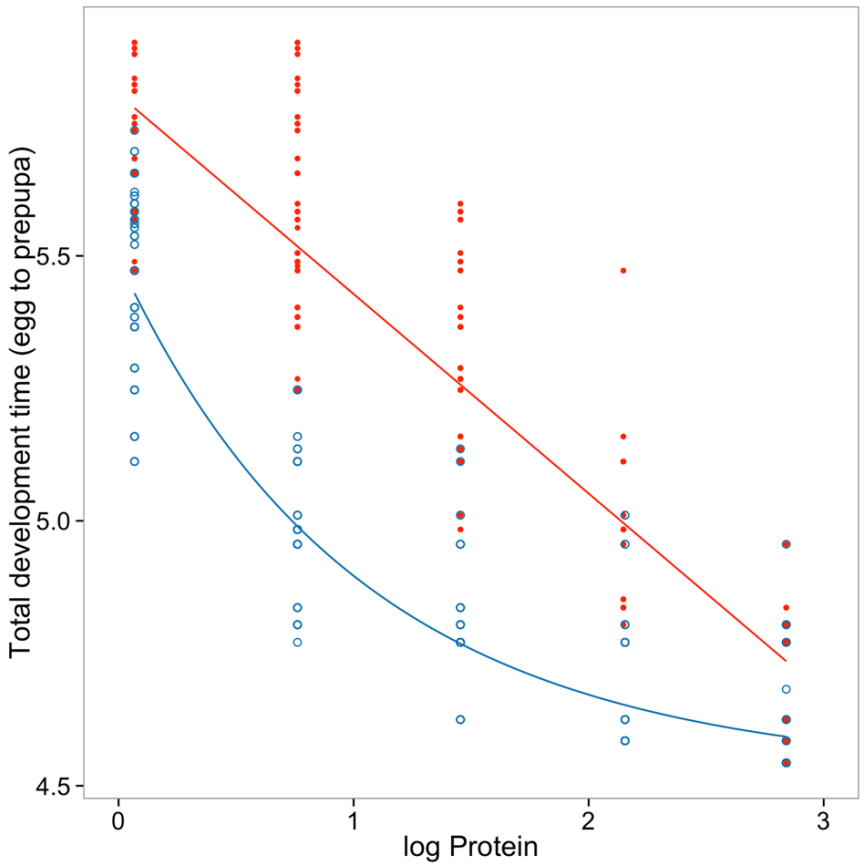

Figure S2 – Effects of the different diets in the development from egg to pupa in *Drosophila melanogaster* larvae. Macronutrient restriction was fit using a General Linear Model and Caloric restriction was fit using a Gompertz Model.

Table S2 – Example of the Least Square means (A) and slopes (B) for differences on female wing size

A)

Treatment	Least square means	SE	df	group
Macro	14.54412	0.01506167	192	1
Caloric	14.55839	0.01506167	192	1

B)

Treatment	logProtein.trend (slope)	SE	df	group
Macro	0.1090628	0.01451699	192	1
Caloric	0.1838491	0.01451857	192	2